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NIGROSTRIATAL DOPAMINE-NEURON FUNCTION FROM NEUROTROPHIC-LIKE PEPTIDE TREATMENT AND NEUROTROPHIC FACTOR DEPLETION

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
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Lexington, Kentucky

Director: Dr. Greg A. Gerhardt, Professor of Anatomy and Neurobiology

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ABSTRACT OF DISSERTATION

NIGROSTRIATAL DOPAMINE-NEURON FUNCTION FROM NEUROTROPHIC-LIKE PEPTIDE TREATMENT AND NEUROTROPHIC FACTOR DEPLETION

Trophic factors have shown great promise in their potential to treat neurological disease. In particular, glial cell line-derived neurotrophic factor (GDNF) has been identified as a potent neurotrophic factor for midbrain dopamine (DA) neurons in the substantia nigra (SN), which lose function in Parkinson's disease (PD). GDNF progressed to phase II clinical trials, which did not meet proposed endpoints. The large size and binding characteristics of GDNF have been suspected to contribute to some of the shortcomings of GDNF related to delivery to target brain regions. Smaller peptides derived from GDNF (Dopamine-Neuron Stimulating Peptides – DNSPs) have been recently investigated and appear to demonstrate trophic-like effects comparable to GDNF. In the described studies, a time course study was conducted to determine *in vivo* DA-release characteristics 1-, 2- and 4- weeks after peptide treatment. These studies determined the effects on DA terminals within striatal sub-regions using microelectrodes. A heterogeneous effect on striatal sub-regions was apparent with the maximum effect in the dorsal striatum – corresponding to terminals originating from the SN.

Dysregulation of GDNF or GDNF signaling is believed to contribute to motor dysfunction in aging and PD. Thus, it is hypothesized that GDNF is necessary for the maintenance and function of neurons. To extend this line of investigation, *in vivo* functional measures (DA-release and -uptake) and behavioral and cellular alterations were investigated in a transgenic mouse model (*Gdnf*$^{+/-}$) with reduced GDNF protein levels. The described studies determined that both DA-uptake and -release properties were altered in middle-aged *Gdnf*$^{+/-}$ mice with only modest reductions in DA neurochemical levels. GDNF levels in *Gdnf*$^{+/-}$ mice were restored to levels comparable to wild-type (WT) counterparts by treatment with GDNF. GDNF protein supplementation led to enhanced motor behavior and increased markers for DA neurons in the SN of *Gdnf*$^{+/-}$ mice. *Gdnf*$^{+/-}$ mice appeared to show a heightened sensitivity to GDNF treatment compared to WT counterparts.

Overall, this body of work examines novel synthetic peptides with potential to enhance DA-neuron function and expands upon the current understanding of GDNF's role in the nigrostriatal pathway.

KEYWORDS: glial cell line-derived neurotrophic factor, dopamine, aging, Parkinson's disease, real-time amperometry

Ofelia M. Littrell

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12/2/2011

For my parents

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Chapter One: Introduction

Dopamine

Dopamine (DA) was first suggested to function as an independent neurotransmitter by Arvid Carlsson (Carlsson, 1959). Carlsson described distinct catecholamine distribution patterns that challenged the existing dogma that DA served exclusively as a metabolic intermediate in the synthesis of norepinephrine and epinephrine. Since the relatively recent acceptance of DA as a chemical messenger in the brain, several DA nuclei have been identified and examined for physiological significance. Indeed, the involvement of DA in reinforcement and reward pathways has led to intense investigation regarding substances of abuse (Sulzer, 2011) as well as other psychiatric disorders like bipolar disorder (Cousins*, et al.*, 2009), schizophrenia (Hirvonen and Hietala, 2011) and attention-deficit hyperactivity disorder (Swanson*, et al.*, 2007).

Arguably, the most widely studied area of neuroscience concerning DA neurotransmission has been its role in motor function within the basal ganglia – specifically concerning the marked loss of functional DA neurons seen in Parkinson's disease (PD) (Bernheimer*, et al.*, 1973). The nigrostriatal pathway is comprised of DA-neuron projections that originate from the midbrain DA nucleus, the substantia nigra (SN), and terminate in the caudate/putamen (striatum). The following studies will focus on the cellular and functional characteristics of DA neurons within the nigrostriatal pathway and associations with motor function in rodent models.

Dopamine Neurotransmission

Synthesis, Storage and Release

Fundamental aspects of DA neurotransmission are depicted in a simplified DA synapse (Figure 1.1). In the presynaptic DA neuron, DA is synthesized from the amino acid tyrosine. Tyrosine is converted to levodopa ((L-3,4-dihydroxyphenylalanine; L-DOPA) in the rate-limiting reaction catalyzed by tyrosine hydroxylase (TH) using a tetrahydrobiopterin (BH_4) co-factor and O_2. Levodopa is converted to DA by aromatic L-amino-acid decarboxylase (AAADC) (Hornykiewicz, 1966). Presynaptic DA may be packaged into acidic vesicles using proton co-transport by the vesicular monoamine transporter (VMAT-2) (Cooper*, et al.*, 2003; Flatmark and Stevens, 1999; Kanner and

Schuldiner, 1987). For most considerations in this dissertation, the term “DA release” is used in reference to depolarization-induced vesicular exocytosis of DA, a Ca^{++}-dependent release process (Cooper*, et al.*, 2003; Grace and Bunney, 1984a), which is a stimulated release event comparable to “phasic” DA release (Goto*, et al.*, 2007). Additionally, single-spike firing activity of DA neurons, also Ca^{++}-dependent, contributes to DA release often referred to as “tonic” DA release (Floresco*, et al.*, 2003; Goto*, et al.*, 2007; Grace and Bunney, 1984b). Both tonic and phasic DA release are relevant to the discussion of DA depletion in PD and related dysfunction of the nigrostriatal pathway (Obeso*, et al.*, 1994). While phasic and tonic DA release are perhaps the most relevant release mechanisms under physiological conditions, carrier-mediated release (Ca^{++}-independent) is another source of increased extracellular DA concentrations (Hurd and Ungerstedt, 1989) used in the described experiments.

Dopamine release, of any mechanism, may be influenced by numerous factors including: the readily releasable pool of DA in the synapse, number/size of vesicles, DA-terminal density and other synthesis- and release-modulating factors (Bark and Wilson, 1994; Gerhardt*, et al.*, 1986; Gillis*, et al.*, 1996; McMillen*, et al.*, 1980; Pothos*, et al.*, 2000; Rosenmund and Stevens, 1996; Santiago and Westerink, 1991; Shui*, et al.*, 1998; Sulzer and Pothos, 2000). Although these aspects of DA-neuron function are relevant in the subsequent studies of DA release and neurochemical changes, a comprehensive treatment of these mechanisms and their interactions is beyond the scope of the current studies. Dopamine-release dynamics are important considerations as the amount of released DA contributes to the net effect on DA receptors. Another contributing factor to the net effect of DA is clearance from the extracellular space (Jones*, et al.*, 1998).

Dopamine Clearance – Transport and Metabolism

Along with the quantity of DA released, another major determinant of the net interaction of DA and DA receptors is the lifetime of DA in the synapse. Because high-affinity uptake by the DA transporter (DAT) is the primary means by which extracellular DA is removed, the DAT is a significant contributor to termination of DA interaction with DA receptors (Iverson, 1975). The DAT is present in highest densities in the striatum (Aquilonius*, et al.*, 1987). Molecular studies indicate there are several sights for glycosylation or phosphorylation that are potentially modulatory (Li*, et al.*, 2004; Ramamoorthy*, et al.*, 2010) and 12 hydrophobic stretches – corresponding to putative transmembrane domains linked by intra- and extra-cellular loops (Giros and Caron,

1993). Decreased function of the DAT has been associated with PD (Gainetdinov*, et al.*, 1997) as well as parkinsonism in aging (Hebert*, et al.*, 1999) – topics discussed further below (see Aging and Dopamine-Neuron Dysfunction: Parkinson's Disease and Parkinsonism). Under physiological conditions, the DAT allows for the transport of DA from the extracellular space to the presynaptic DA terminal (Figure 1.1) (Giros*, et al.*, 1996; Jones*, et al.*, 1998) via co-transport of Na^+ and Cl^- (Reith*, et al.*, 1997; Wheeler*, et al.*, 1993). Under some conditions, pharmacological manipulation or ionic disturbance may facilitate the reverse transport of DA from the cytosol to the extracellular space (Seiden*, et al.*, 1993; Sulzer, 2011; Wheeler*, et al.*, 1993).

Although the transport of DA by the DAT is generally considered the primary means of DA clearance, particularly in brain areas of high DAT density like the striatum (Aquilonius*, et al.*, 1987; Cass*, et al.*, 1993), enzymatic degradation and dilution of synaptic DA by diffusion are also considerations regarding DA neurotransmission (Cooper*, et al.*, 2003). Although both metabolism and diffusion are minor contributors to DA clearance in the striatum, for the purposes of these studies degradation by metabolism will be discussed further in order to clarify the possible sources of metabolites (Cass and Gerhardt, 1995; Cass*, et al.*, 1993).

Metabolism of DA is catalyzed by three primary enzymes: monoamine oxidase (MAO) and COMT (catechol-*o*-methyl transferase) (Westerink, 1985) (Figure 1.1). Two forms of MAO exist (MAO-a and MAO-b) (Schoepp and Azzaro, 1982). The action of either enzyme results in aldehyde conversion of DA and further processing of the aldehyde intermediate by aldehyde dehydrogenase (AD) (Knoll and Magyar, 1972). For the purposes of these studies MAO-a and MAO–b forms should be differentiated primarily based on their localization in the brain, where MAO-a is primarily intraneuronal and MAO-b is extraneuronal, although other distinctions exist (Waldmeier, 1987). The enzyme, COMT, is present extraneuronally (Broch and Fonnum, 1972; Kaplan*, et al.*, 1979). Extracellular DA may be converted to 3,4-dihydroxyphenylacetic acid (DOPAC) through the combined actions of MAO-b and AD. Cytosolic (non-vesicularized) DA may be converted to DOPAC by MAO-a and AD. Cytosolic DOPAC may diffuse to the extracellular space where it is a substrate for COMT to produce homovanillic acid (HVA) (Kopin, 1985). Thus, metabolism of DA produces two major metabolites, DOPAC and HVA, which may be referred to as primarily intracellular and extracellular metabolites, respectively (Roffler-Tarlov*, et al.*, 1971). A third metabolic product, 3-methoxytyramine (3-MT) may also be formed from the interaction of COMT with extracellular DA.

Although, 3-MT is considered a minor metabolite (Westerink and Spaan, 1982) because it is readily converted to DOPAC by MAO-b (Kopin, 1985). It is also relevant to clarify that HVA is the primary metabolite in primates (Sharman*, et al.*, 1967; Sternberg*, et al.*, 1983) while DOPAC and HVA are present in comparatively equal quantities (Dedek*, et al.*, 1979) in the rodent brain with a slight preference for DOPAC.

Dopamine Receptors

Extracellular DA may bind to DA receptors present on presynaptic and postsynaptic targets, which are broadly classified into two families: D_1 or D_2 (Cooper*, et al.*, 2003; Kebabian and Calne, 1979; Kebabian and Greengard, 1971; Vallone*, et al.*, 2000) (Figure 1.1). The two families of DA receptors are based on the stimulatory (D_1) or inhibitory (D_2) effect on adenylate cyclase (AC) (Beaulieu and Gainetdinov, 2011). Additionally, several subdivisions of DA receptors exist based on biochemical, structural and pharmacological distinctions: D_1-class (D_1, D_5) (Tiberi*, et al.*, 1991), D_2-class (D_2, D_3, D_4) (Vallone*, et al.*, 2000). The stimulatory action on AC following stimulation of D_1 receptors (a $G\alpha_{s/olf}$ family of G-proteins) activates the second messenger cAMP and protein kinase A (PKA) (Beaulieu and Gainetdinov, 2011). In a simplified and generalized signaling paradigm, D_1-receptor activation leads to DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M(r) 32 kDa) phosphorylation and activation, Activity of DARPP-32 modulates: ionotropic glutamate and GABA receptors, specific ion channel sensitivity and protein phosphatase 1 (PP1) (Bateup*, et al.*, 2008; Beaulieu and Gainetdinov, 2011; Greengard, 2001; Hemmings*, et al.*, 1984; Svenningsson*, et al.*, 2004). Stimulation of D_2-class receptors, which couple to the $G\alpha_{i/o}$ family of G-proteins, cause inhibition of AC (Vallone*, et al.*, 2000). Generally, D_2-class receptors have the opposing effect on intracellular signaling processes compared with those outlined for the D_1-class receptors. The D_1-class of DA receptors are present, exclusively, on postsynaptic targets while D_2-class receptors may be present both presynaptically (autoreceptors) and postsynaptically (De Mei*, et al.*, 2009). Presynaptically, D_2-class autoreceptors may modulate numerous aspects of neuron function including synthesis, release and uptake (Cass and Gerhardt, 1994; De Mei*, et al.*, 2009; Kehr*, et al.*, 1972; Sibley, 1999; Vallone*, et al.*, 2000).

Following this brief overview of the functional DA synapse, the role of DA in movement disorders like Parkinson's disease (PD) and age-associated parkinsonism

must be addressed in the context of the basal ganglia – the motor initiating and coordinating centers of the brain (Kandel*, et al.*, 2000).

The Basal Ganglia

In the mammalian brain the major dopaminergic pathways are identified as: nigrostriatal (A9), mesolimbic and mesocortical (A10), and tuberoinfundibular (A8) (Cooper*, et al.*, 2003; Dahlstrom and Fuxe, 1964a). Because the focus of these studies is on the nigrostriatal pathway, its coordinative function with other brain nuclei of the basal ganglia is described further. Although some components of the basal ganglia have been associated with cognitive, emotional and other functions (Afifi, 1994; Afifi, 2003), this section will focus on the nuclei and projections most relevant to motor planning, initiating and coordinating. For a more comprehensive review of basal ganglia circuitry see (Albin*, et al.*, 1989; Alexander*, et al.*, 1986; Mink, 2007).

The basal ganglia consist of four subcortical nuclei that are divided into interconnected sub-structures (depicted schematically; Figure 1.2): the striatum, globus pallidus (GP), substantia nigra (SN) and subthalamic nucleus (STN). The GP may be divided into separate anatomical and functional divisions: internal (GPi) and external (GPe) segments. Similarly, the SN is differentiated into the *pars compacta* (SNc) and *pars reticulata* (SNr) (see The Nigrostriatal Pathway). In primates, the striatum is further described as two separate nuclei, the caudate nucleus and putamen, divided by the internal capsule. Because the rodent brain lacks delineation of the caudate nucleus and putamen, the striatum is the accepted nomenclature (Bentivoglio and Morelli, 2005; Webster, 1975). The action of DA in the striatum is directed to γ-aminobutyric acid (GABA)-producing medium spiny neurons (MSNs) (Gerfen, 1992a). Dopamine acts on D_1- and D_2-class receptors on MSNs projecting to the GPi/SNr (direct pathway) and GPe (indirect pathway), respectively. It is likely that this view of DA-receptor class segregation on MSNs is inaccurate. Rather than restricted expression of only one receptor class on individual MSNs, there is evidence for co-localization of D_1 and D_2 receptors with proportional differences between the two in individual neurons and other mechanistic differences that contribute to the cellular pathways activated (Waszczak*, et al.*, 1998; Weiner*, et al.*, 1991). For our purposes, the MSN sub-types will be considered D_1- or D_2-"dominant" types, both GABA producing, which may be distinguished based on projection targets and specific protein expression: dynorphin and substance p (D_1), enkephalin (D_2) (Albin*, et al.*, 1989). In the direct pathway, striatal inhibitory projections

are stimulated by DA (D_1 dominant) and inhibit the GABAergic projections to the thalamus from the GPi and SNr. In the indirect pathway, inhibitory projections from the GPe are active leading to inhibition of excitatory projections from the STN to GPi/SNr and inactivation of inhibitory projections to the thalamus from the GPi/SNr. Because thalamic output to motor cortices is increased and decreased through the direct and indirect pathways, respectively, the net effect of DA (increasing activity in the direct pathway and decreasing activity of the indirect pathway) is to facilitate glutamatergic output in the thalamocortical projection (Figure 1.2)

The Nigrostriatal Pathway

The nigrostriatal pathway contains dopaminergic projections originating from the midbrain DA nucleus – the SN. Dopamine neurons of the SN project anteriorly and diffusely to the striatum (Lindvall and Bjorklund, 1974; Veening*, et al.*, 1982). The SN is subdivided into the dense band of pigmented, neuromelanin-containing (Zecca*, et al.*, 2001), DA-neuron cell bodies (the *pars compacta* (SNc)) and a more ventral region containing fibers from the SNc but proportionately fewer DA-neuron cell bodies – the SN *pars reticulata* (SNr) (Atherton and Bevan, 2005; Bentivoglio and Morelli, 2005; Francois*, et al.*, 1987).

The striatum is considered a highly heterogeneous structure for several reasons. Regarding DA innervation of the striatum, afferents arise from several nuclei including the SNc (A9) and ventral tegmental area (VTA, A10) (Dahlstrom and Fuxe, 1964b), which demonstrate distinct innervation patterns and functional characteristics (Cline*, et al.*, 1995; Fallon*, et al.*, 1978; Langer and Graybiel, 1989; Lindvall and Bjorklund, 1974). The striatum may also be subdivided into functional components based on innervation patterns unique to the motor- (dorsolateral), associative- (medial) and limbic-striatum (ventral), which are innervated by the motor cortices, associative/prefrontal cortices and limbic structures, respectively (Joel and Weiner, 2000; McGeorge and Faull, 1989). This functional organization is superimposed on an additional organization of the striatum into striosomes (patch) and matrix components. Patch and matrix regions are discriminated by differential patterns of acetylcholinesterase staining, presence of specific peptides and connections with other brain regions (Gerfen, 1985; Gerfen, 1992b; Gerfen*, et al.*, 1987a; Gerfen*, et al.*, 1987b).

The primary cell type (~95%) in the striatum is the GABA producing MSN. Medium spiny neurons, distributed throughout the striatum, give rise to the striatonigral

(direct pathway) or striatopallidal projections (indirect pathway) (Bentivoglio and Morelli, 2005; Lobo, 2009). The remaining cells in the striatum are interneurons of the cholinergic or GABAergic type, which may be modulated by DA and other neurotransmitters (Gittis*, et al.*, 2010; Partridge*, et al.*, 2002; Tozzi*, et al.*, 2011; Vincent*, et al.*, 1983).

Aging and Dopamine-Neuron Dysfunction: Parkinson's Disease and Parkinsonism

Age is the most substantial risk factor for developing PD of the non-familial/idiopathic type (Bennett*, et al.*, 1996; Collier*, et al.*, 2011; Fearnley and Lees, 1991), which is typically diagnosed upon emergence of clinical symptoms in the 6th decade of life or beyond (Khealani and Baig, 2006). Although early-onset forms of PD exist, which are associated with specific genes (Dawson and Dawson, 2003; Schiesling*, et al.*, 2008; Vila and Przedborski, 2004), they represent a small proportion of total PD cases (~5%) (Cookson*, et al.*, 2005; Dauer and Przedborski, 2003; Davie, 2008). Genetic studies of early-onset PD have helped to link several genes to the more common, aptly-named, idiopathic type (Cookson*, et al.*, 2005; Illarioshkin*, et al.*, 2007; Sharma*, et al.*, 2011), which has a poorly understood pathoetiology. Some of the contributing factors to the development of idiopathic PD will be discussed. For the purposes of this work, the remaining discussion will be directed at the idiopathic (sporadic) type.

The role of DA in the basal ganglia has been studied extensively and implicated in movement disorders, particularly regarding loss of DA neurons in the SN (Adams and Victor, 1977; Bennett*, et al.*, 1996; Fearnley and Lees, 1991; Hoehn and Yahr, 1967; Hornykiewicz and Kish, 1987). Parkinson's disease involves the progressive and selective loss of midbrain DA neurons in the SN with a consequent loss of DAergic input to the striatum (Bernheimer*, et al.*, 1973; Fearnley and Lees, 1991; McGeer*, et al.*, 1988). Clinically, PD is characterized by cardinal motor symptoms (bradykinesia, rigidity, postural instability and tremor (Hoehn and Yahr, 1967)) caused, in part, by increased activity in the indirect pathway and decreased activity in the direct pathway, which contribute to reduced thalamic output to the motor cortices (Mink, 2007). Of note, a definitive diagnosis of PD can only be made *post mortem* to confirm defining pathological features: gross depigmentation patterns of DA neurons and cytoplasmic inclusion (Lewy) bodies (Foltynie*, et al.*, 2002; Jellinger, 1999).

Although other DA neurons and neurotransmitter systems are dysfunctional in PD (Barone, 2010; Braak*, et al.*, 2003a; Huot*, et al.*, 2011), perhaps the most striking pathological feature is the characteristic degeneration of DA neurons in the SN. The relative selectivity of degeneration in the SNc proper (Fearnley and Lees, 1991; Uhl*, et al.*, 1985) has prompted more intense examination of properties of these neurons for insights into PD etiology. Prevailing theories on cell death in PD include: oxidative damage (Hald and Lotharius, 2005; Jenner and Olanow, 1996), mitochondrial dysfunction (Abou-Sleiman*, et al.*, 2006) and abnormal protein aggregation (Golbe, 2003) related to α-synuclein (the main constituent of Lewy bodies) (Spillantini*, et al.*, 1997). These proposed processes are conceivably not mutually exclusive and interact with multiple genetic and environmental factors (Braak*, et al.*, 2003b; Choi*, et al.*, 2006; Chung*, et al.*, 2001; Greenamyre and Hastings, 2004). It is proposed that an intrinsic property of DA neurons, containing DA, leads to oxidative damage due to the auto-oxidation of cytosolic DA to the reactive quinone (Greenamyre and Hastings, 2004; Sulzer and Zecca, 2000). Quinone formation may lead to increased generation of reactive oxygen species that deleteriously affect mitochondrial function. Mitochondrial dysfunction may cause additional oxidative stress, cellular damage/death (Berman and Hastings, 1999; Jana*, et al.*, 2011) and abnormal protein interactions that contribute to α-synuclein-associated neurotoxicity (Lewy-body deposition) (Conway*, et al.*, 2001). Nigrostriatal DA neurons generate action potentials in “pacemaker”-like continuous patterns that have been suggested to cause increased energetic demands leading to mitochondrial stress (Surmeier, 2007) – another potential source of reactive oxygen species generation and oxidative stress that contribute to Lewy-body formation and neurodegeneration (Greenamyre and Hastings, 2004). Additionally, the DAT has been suggested to predispose nigrostriatal DA neurons to injury because of a relatively high density of DAT expression (Cass and Gerhardt, 1995; Shimada*, et al.*, 1992; Storch*, et al.*, 2004). Thus, increased cytosolic DA transported from the extracellular space by the DAT may auto-oxidize – forming reactive quinones (Sulzer and Zecca, 2000). The DAT also plays a critical role in facilitating cellular entry of toxins (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Gainetdinov*, et al.*, 1997) or 6-hydroxydopamine (6-OHDA)) used to experimentally induce DA-neuron death (Storch*, et al.*, 2004). Thus, the DAT may increase susceptibility of DA neurons to existing or unknown environmental toxins (Cicchetti*, et al.*, 2009; Opeskin and Anderson, 1997).

Relationships between PD and aging are muddled because of the observed motor impairment commonly associated with aging termed “age-associated parkinsonism”, a condition that can be difficult to differentiate from sub-clinical PD (Bennett*, et al.*, 1996). Parkinsonism is also used in the literature to refer to sub-types of movement disorders (including primary PD) as well as experimental models of PD (Baldereschi*, et al.*, 2000; Christine and Aminoff, 2004; Kitayama*, et al.*, 1992; Opeskin and Anderson, 1997; Samaranch*, et al.*, 2010). Although PD is considered a disease of aging, the specific pattern of neurodegeneration seen in subdivisions of the SNc contrasts the loss seen in the aged (non-PD) brain (Fearnley and Lees, 1991; Kish*, et al.*, 1992). The pattern distinctions suggest that PD should not be considered an accelerated-aging disease of DA neurons (Fearnley and Lees, 1991). The striking loss of functional DA neurons at the onset of PD (~60%)(Bernheimer*, et al.*, 1973) also contrasts the neurodegeneration seen in aging (7-33%)(Fearnley and Lees, 1991; Hirai, 1968; McGeer*, et al.*, 1977), suggesting that more understated changes in neuron function produce age-associated parkinsonism. There is substantial variability in the reported degree of degeneration in aging because of differences in defined anatomical boundaries, inclusion/exclusion of individuals with existing neurodegenerative disease or sub-clinical PD as well as stereological methods (Stark and Pakkenberg, 2004). Some studies suggest very little neurodegeneration accompanies normal aging (Kubis*, et al.*, 2000). It is proposed that changes in more dynamic properties of DA-release and -uptake lead to age-associated parkinsonism in animal models (Gerhardt*, et al.*, 1995; Hebert and Gerhardt, 1998; Hebert and Gerhardt, 1999; Yurek*, et al.*, 1998) and humans (Volkow*, et al.*, 1998a; Volkow*, et al.*, 1994).

In the case of primary PD, current medical management involves a combination of drug therapies that typically act by modulating DA -synthesis, -metabolism and/or -receptor activation (Djaldetti and Melamed, 2002; Fahn*, et al.*, 2004; Goudreau, 2006; Salawu*, et al.*, 2010; Yamada and Yasuhara, 2004). When pharmacological therapies are not effective, surgical therapies may re-establish balance through selective lesioning or stimulation of non-DAergic nuclei within the basal ganglia (Okun and Vitek, 2004). Current medical and surgical management approaches are only symptomatic treatment strategies and do not slow disease progression. The treatment of underlying neurodegeneration to slow disease progression is one of the great prospects of neurotrophic factor use in PD (Chiocco*, et al.*, 2007; Dauer, 2007; Peterson and Nutt, 2008).

Trophic Factors

In the most general and basic terms, trophic factors are described as naturally occurring proteins with survival- and function -enhancing effects. The term “neurotrophic factor” has been used interchangeably with trophic factor when biological effects are concerning nerve cells (Levi-Montalcini, 1987; Peterson and Nutt, 2008). The first trophic factor shown to affect nerve cells was identified in the 1950s, nerve growth factor (NGF) (Cohen*, et al.*, 1954). Since the discovery of the first neurotrophic factor, additional neurotrophic factors, families and sub-families have been identified that are relevant to the treatment of PD and other neurodegenerative diseases (Chiocco*, et al.*, 2007; Dauer, 2007; Peterson and Nutt, 2008).

Glial Cell line-Derived Neurotrophic Factor: A Potent Trophic Factor for Midbrain Dopamine Neurons

Trophic factors have shown promising results in the treatment of PD (Peterson and Nutt, 2008). Perhaps the most promising and well-characterized neurotrophic factor concerning DA neurons is glial cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor-β (TGF-β) superfamily (Lin*, et al.*, 1993; Saarma, 2000). Positive behavioral, histological and neurochemical effects have been demonstrated in the aged rodent (Hebert and Gerhardt, 1997) and non-human primate (Ai*, et al.*, 2003; Grondin*, et al.*, 2003; Maswood*, et al.*, 2002) following GDNF treatment. Although the enhancing effects on DA neurons by GDNF is applicable to aging and parkinsonism, the majority of the discussion following will focus on GDNF studies in primary PD.

Following numerous studies showing modulation of DA systems by GDNF (Georgievska*, et al.*, 2002; Grondin*, et al.*, 2003; Grondin*, et al.*, 2002; Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996; Lindvall and Wahlberg, 2008; Rosenblad*, et al.*, 1996), GDNF progressed to clinical trials. Because of the large size and other biochemical characteristics of neurotrophic factors, blood brain barrier permeability is unlikely (Pardridge, 2003) and intracerebral catheter administration is a necessary route to reach target brain areas and maintain protein integrity (Thorne and Frey, 2001). Based on non-human primate studies demonstrating significant behavioral improvement after GDNF delivery to the ventricles (Gash*, et al.*, 1996; Zhang*, et al.*, 1997), GDNF was delivered to the ventricles in a double-blind clinical trial. Trials using ventricular

delivery produced adverse side effects and failed to show significant clinical benefit – a finding attributed to inadequate penetrance to target brain areas (Kordower*, et al.*, 1999; Nutt*, et al.*, 2003). Although small open-label trials using direct delivery to the putamen improved motor scores measured by the Unified Parkinson's Disease Rating Scale (UPDRS) (Gill*, et al.*, 2003; Slevin*, et al.*, 2005), a larger placebo-controlled trial did not meet proposed endpoints (25% improvement)(Lang*, et al.*, 2005). Initial results were promising, however, placebo-controlled trials were halted due to safety concerns: the presence of neutralizing antibodies in some patients (Tatarewicz*, et al.*, 2007) and indications of cerebellar toxicity in non-human primate studies (Hovland*, et al.*, 2007) – a finding not reproduced in patients (Chebrolu*, et al.*, 2006)). The trials are viewed as highly controversial with numerous conclusions and mixed opinions. There are reservations about the design of the placebo-controlled study and interpretation of safety issues (Hutchinson*, et al.*, 2007; Matcham*, et al.*, 2007; Penn*, et al.*, 2006). Also, lack of consistency in several important variables (e.g. catheter type/infusion method and dose) makes interpretation problematic (Sherer*, et al.*, 2006).

Following the GDNF clinical trials, it has been suggested that limited diffusion to the preferred brain regions underlies some of the shortcomings (Salvatore*, et al.*, 2006) (Salvatore*, et al.*, 2003). Although neurotrophic factors are promising candidates for the treatment of neurodegenerative disease, the considerations for delivery to the CNS are daunting, if not limiting (Peterson and Nutt, 2008; Thorne and Frey, 2001). The delivery obstacles associated with trophic factor delivery to the brain have prompted the search for smaller mimetic molecules with trophic or trophic-like effects and greater potential for modification to improve bioavailability (Bespalov and Saarma, 2007).

Small Peptides as Trophic-like Molecules

The inherent bioavailability limitations of large trophic molecules (e.g large size and heparin-binding characteristics)(Salvatore, et al., 2006) stimulated interest in smaller biologically-active molecules that may provide comparable therapeutic benefit and avoid some specific delivery issues (Beglova, et al., 2000; Bespalov and Saarma, 2007; Maliartchouk, et al., 2000; Peleshok and Saragovi, 2006; Saragovi and Gehring, 2000).

In the case of GDNF, these small molecules may come in the form of pro-peptides – peptides derived from the pro-sequence (Bradley, et al., 2010; Immonen, et al., 2008; Kelps, et al., 2011). GDNF and other trophic molecules are processed as immature precursors that are be cleaved to yield the mature trophic molecule and pro-

protein(s) (Chao and Bothwell, 2002; Lee, et al., 2001). The role of the pro-protein(s) was thought to be limited to regulation of folding and secretion of the mature secreted protein (Gray and Mason, 1990). Recently, distinct signaling actions of uncleaved trophic factors (pro-neurotrophins) have been identified for brain-derived neurotrophic factor (BDNF) (pro-BDNF) and NGF (pro-NGF) (Ibanez, 2002; Nykjaer, et al., 2004; Teng, et al., 2005) that are unique from the signaling actions of the mature trophic factor alone. There is also increasing evidence for conservation of pro-protein sequences among related species (Bradley, et al., 2010; Lee, et al., 2001). Taken together, these findings suggest novel roles and possible physiological actions of pro-peptides that may act as more than just chaperones (Bradley*, et al.*, 2010). The comparatively small size and less complex structure of pro-protein peptides increases their clinical utility because they may be more easily modified and are typically stable – making delivery and peptide manipulation to increase bioavailability more feasible for clinical applications (Thorne and Frey, 2001).

Of particular interest, two synthetic peptides (of 5- and 11- amino acids) are predicted to be generated from the proteolytic processing of the pro-sequence of pro-GDNF. The human and rodent forms of these pro-peptides have been investigated by our group and others (Bradley, et al., 2010; Immonen, et al., 2008; Kelps, et al., 2011). Notably, the peptides have been investigated for their effect on DA neurons *in vitro* and *in vivo*. Recent studies show evidence for considerable biological activity relevant to DA-neuron dysfunction in PD/parkinsonism (Bradley, et al., 2010; Kelps, et al., 2011). The smaller size makes these pro-peptides excellent candidates for modification to administer by oral or intranasal routes (Born, et al., 2002). Nasal or oral administration is a favorable alternative to intracranial delivery or gene therapy approaches, which are required for GDNF delivery (Bjorklund, et al., 2000; Gill, et al., 2003; Grondin, et al., 2002; Patel and Gill, 2007).

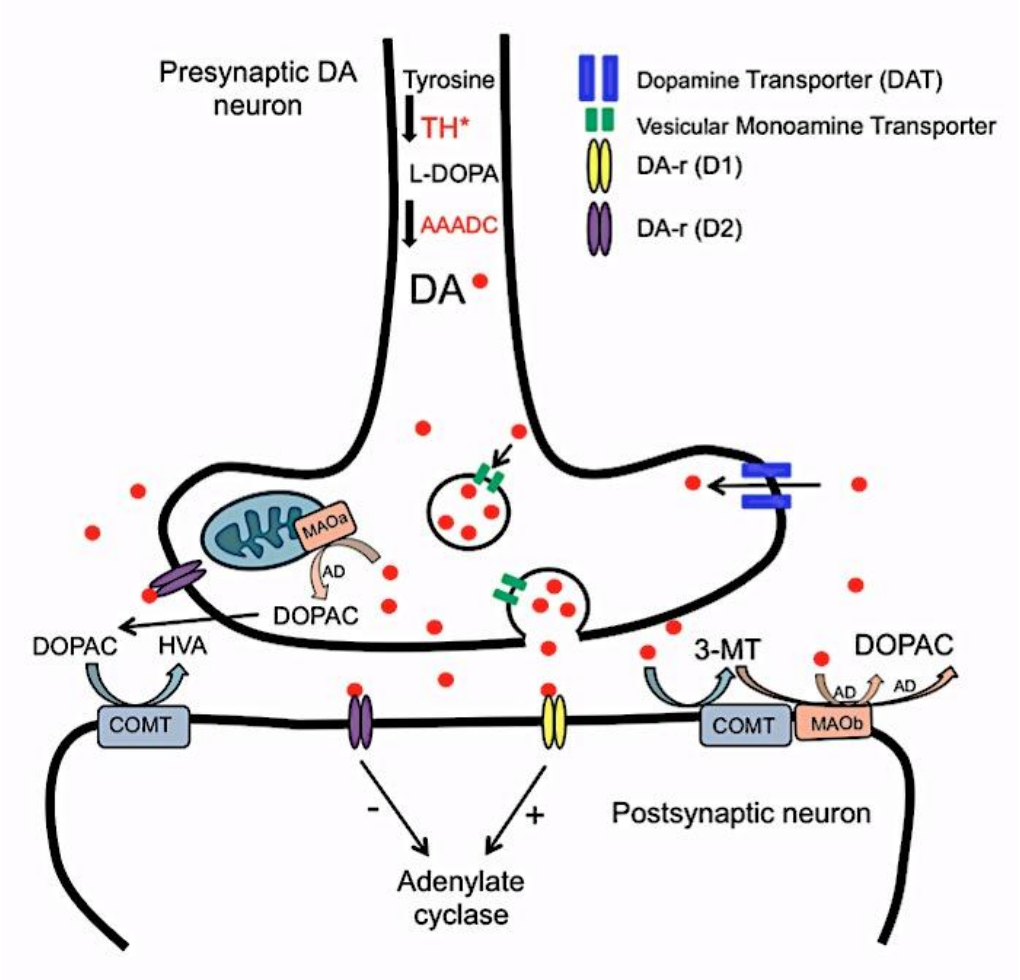

Figure 1.1 Schematic of a Dopamine Synapse

Several aspects of DA neurotransmission are depicted in this simplified and generalized dopamine (DA) synapse b. Dopamine is synthesized in the presynaptic neuron by the conversion of tyrosine to levodopa (L-DOPA) by tyrosine hydroxylase (TH), the rate-limiting enzyme in DA synthesis. Dopamine is produced by the actions of aromic amino-acid decarboxylase (AAADC) on L-DOPA. Intracellular DA may be vesicularized via the vesicular monoamine transporter (V-MAT) in the presynaptic neuron and released in a Ca^{++}-dependent mechanism upon membrane depolarization sufficient to reach an action potential. Cytosolic DA (non-vesicularized) may be metabolized by an intracellular enzyme, monoamine oxidase (MAO-a), localized to the outer membrane of the mitochondria. Dopamine released to the extracellular space is metabolized by MAO-b and/or catechol-*o*-methyltransferase (COMT) to generate metabolic products: 3,4-

dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and a less prevalent intermediate in the CNS 3-methoxytyramine (3-MT). Extracellular DA is removed from the extracellular space by the Na^+-Cl^--dependent DA transporter (DAT). Extracellular DA may bind to DA receptors, which are broadly classified into D_1- and D_2-subtypes based on stimulatory or inhibitory effects on adenylate cyclase (AC). Both subtypes are present on the postsynaptic neuron. Dopamine receptors present on the presynaptic neuron, autoreceptors (D_2), are also targets for DA action and may modulate DA -release and -synthesis. Figure modified from Cooper *et al.,* 2003.

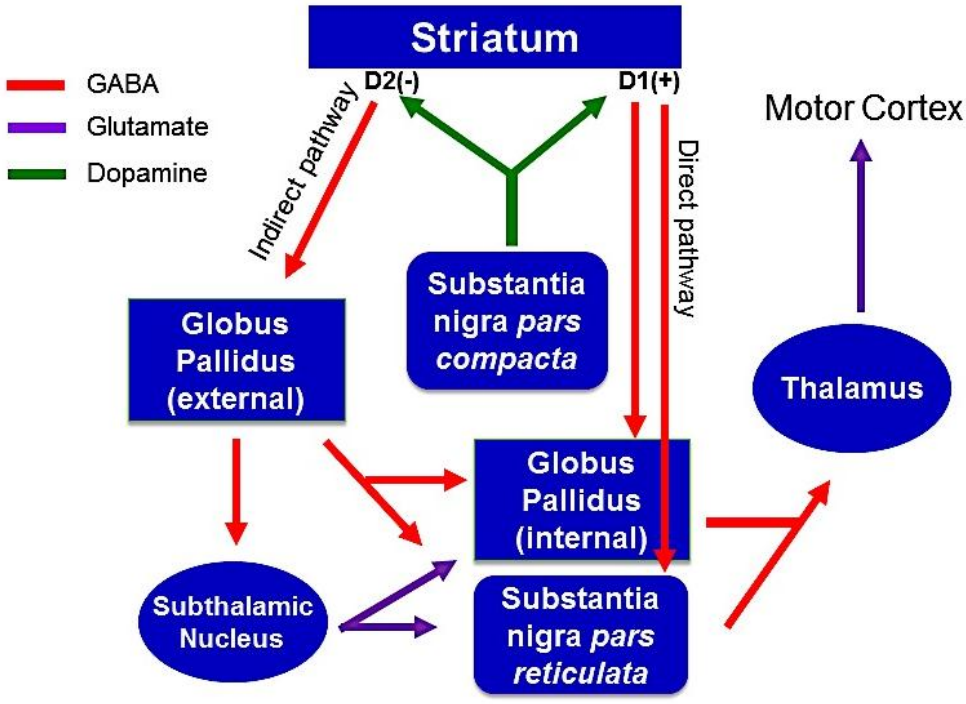

Figure 1.2 Schematic of Basal Ganglia

A simplified schematic diagram of components and connections of the basal ganglia is shown. Dopamine innervation of the striatum arises from midbrain DA-neuron cell bodies in the SNc - the nigrostriatal projection. Activity within the basal ganglia is discussed using distinctions between the direct and indirect pathway, where DA innervation increases and decreases activity in each pathway, respectively. Specifically, activation of D_1 receptors (direct pathway) leads to activation of inhibitory (GABA) fibers projecting from the striatum to the GPi and SNr. Inhibitory projections from the GPi and SNr project to the thalamus. The action of DA innervation of the striatum causes inhibitory projections to the thalamus to be inactivated (disinhibition). Thus, excitatory projections from the thalamus to the motor cortices are active. In the indirect pathway, DA activation of D_2 receptors in the striatum decreases activity of inhibitory projections from the striatum to the GPe. Projections from the GPe inhibit GABAergic projections from the GPi/SNr to the thalamus – allowing excitatory thalamic output to the motor cortices. The GPi/SNr may also be activated by excitatory glutamate projections from the STN, which are also under inhibitory control of the GABAergic projections from the GPe through the indirect pathway. Thus, decreased activity in the indirect pathway results from DA stimulation of D_2 receptors – effectively increasing thalamic output to the motor cortices.

Abberviations used: substantia nigra pars compacta (SNc), globus pallidus [GP, internal segment (GPi), external segment (GPe)], substantia nigra *pars compacta* (SNc), substantia nigra *pars reticulata* (SNr), subthalamic nucleus (STN), γ-aminobutyric acid (GABA). Figure modified from Kandel *et al.,* 2000.

Thesis Outline

In these studies the effect of trophic factors and trophic-like molecules were investigated in the context of nigrostriatal DA-neuron function and movement disorders – topics highly concerned with PD and age-associated parkinsonism. In Chapter Three, the effects of synthetic phenotypic-mimetic trophic peptides – DNSPs – were investigated with attention to evoked DA release and tissue neurochemical content after a single peptide treatment to the SN. In particular, an 11-amino-acid amidated peptide derived from the pro-region of glial cell line-derived neurotrophic factor (GDNF) was investigated. After several experiments that have shown enhanced DA-neuron function associated with this peptide, the peptide has been named Dopamine-Neuron Stimulating Peptide-11 (DNSP-11). These studies were designed to test the overarching hypothesis: DNSP-11 enhances dynamic properties of DA-neuron function in the nigrostriatal pathway. Specifically, sub-regional effects of DNSP-11 on DA release in the striatum were investigated along with the time course of effects – investigating DA release 1-, 2- and 4-weeks after a single treatment to the SN. The most exciting finding of these studies is the enhanced striatal DA release after treatment with DNSP-11, a finding that suggests enhanced function of DA neurons in the SN. Co-injection of DNSP-11 and a related peptide, Dopamine-Neuron Stimulating Peptide-5 (DNSP-5), was investigated to determine if the related pro-peptides exhibited synergistic effects on DA neurochemical function. Interestingly, the enhancing effects on extracellular neurochemical levels, as seen previously with DNSP-11 treatment alone, were not reproduced. Whole tissue dopamine neurochemistry, however, was enhanced after treatment with the peptide mixture – a finding not seen with DNSP-11 treatment alone. Although the functional relationship between related peptides derived from the GDNF pro-sequence is unclear, we believe DNSP-11 and possibly other synthetic peptides are promising therapeutic candidates for DA-neuron -degeneration and -dysfunction (e.g. Parkinson's disease).

In Chapter Four, dynamic properties of DA-neuron function were characterized in a unique transgenic mouse model with a partial GDNF depletion – having a single functional GDNF allele (*Gdnf*$^{+/-}$). Middle aged, 12-month-old, *Gdnf*$^{+/-}$ mice have demonstrated diminished spontaneous locomotor activity and enhanced synaptosomal DA uptake compared to wildtype (WT) mice. In these studies, the following hypothesis was tested: GDNF depletion causes *in vivo* alterations in DA-neuron function that contribute to locomotor function. In the presented studies, specific functional indices of DA-neuron function – DA-uptake and -release – were investigated *in vivo*. The major

finding of these studies is that DA uptake by the DAT appears to contribute to motor deficits demonstrated in the *Gdnf*$^{+/-}$. Other considerations that may influence the behavioral phenotype of *Gdnf*$^{+/-}$ mice were investigated: DA-receptor expression and DA--synthesis and -metabolism. A slight decrease in tissue levels of DA was indicated as well as upregulation of mRNA and protein levels of the DA D_2-receptor. Altogether, these studies characterize a unique mouse model of neuron dysfunction caused by a GDNF protein reduction. The findings indicate an important role of GDNF in aging and nigrostriatal dysfunction.

In Chapter Five, the use of GDNF supplementation in age-related changes in DA-neuron function was investigated in *Gdnf*$^{+/-}$ mice. The hypothesis that DA-neuron deficiencies and related behavioral impairments are reduced by GDNF treatment was tested. To determine if age-related DA-neuron abnormalities were reversible, GDNF was administered to the striatum of 12-month-old *Gdnf*$^{+/-}$ mice and similar indices of DA-neuron function were investigated as outlined in Chapter Four. These studies further investigated the consequences of trophic factor depletion and examined the function of trophic factor treatment to ameliorate abnormalities in nigrostriatal DA-neuron function. Notably, markers for functional DA neurons and locomotor activity were increased in *Gdnf*$^{+/-}$ mice and were comparable to WT counterparts.

Chapter Two: Materials and Methods

Reagents

Most reagents were purchased from Sigma-Aldrich (St. Louis, MI). Exceptions are noted.

Animals and Procedures

Ethics Statement

Protocols for animal care were in agreement with NIH approved guidelines and compliant with local institutional protocols at the University of Kentucky Medical Center (rats) and the Medical University of South Carolina (mice). Procedures were in strict agreement with the *Guide for the Care and Use of Laboratory Animals.* Surgical procedures followed AALACI guidelines and were approved by the Institutional Animal Care and Use Committee.

Animal Housing and Husbandry

Animals were maintained under a 12:12 hour light:dark cycle with food and water available *ad libitum* at an ambient temperature of 20-22° C. Procedures and experiments were performed during the light cycle.

Rat Studies

Fischer 344 (F344) rats were obtained from Harlan Laboratories Inc. (Indianapolis, IN). All rats were 3-6 months of age. Rats were housed 1 per cage in the University of Kentucky animal facilities.

Mouse Studies

A nonfunctional GDNF allele was generated by replacing part of the GDNF protein encoding exon, exon 3, with a selectable marker neomycin phosphotransferase-expressing cassette. Generation and genotyping of *Gdnf*$^{+/-}$ mice is described in detail in previous work (Pichel*, et al.*, 1996). Transgenic mice and non-transgenic littermate control mice were obtained from a colony established at the Medical University of South Carolina. Mice were bred on a C57Bl/6J background in agreement with NIH-approved protocols. After transfer to the University of Kentucky, mice were acclimated for a

minimum of 1 week before use in experiments or surgical procedures. Mice were housed 3-4 per cage. Male *Gdnf*$^{+/-}$ mice (12-months-old) were compared with age-matched WT littermates in all experiments. Age-matched C57/BL6 mice (non-littermate/non-transgenic) (Charles River Laboratories, Wilmington, MA) were used as an additional control group in some experiments (see Chapter Five: GDNF Replacement in Gdnf+/- Transgenic Mice).

Intracranial Drug Delivery - Survival Surgical Procedures

Instruments used for survival procedures were sterilized and aseptic techniques were followed using a laminar flow hood to maintain aseptic working conditions. Large fixed equipment and instruments that were not autoclave-compatible were disinfected with 70% ethanol and/or covered with a sterile drape. Animals used for survival surgical procedures were anesthetized with isoflurane gas (1.5-2.5% in O_2) and positioned in a stereotaxic frame for the rat or mouse (Kopf Instruments, Tujunga, CA, USA). A craniotomy was performed for access to the targeted structures and drugs were delivered using a 26-gauge needle (cannula only) (22026-01, point style-3; Hamilton Company, Reno, NV) attached to a 25-µL Hamilton syringe (80408, point style 3; Hamilton Company, Reno, NV) using plastic tubing (Zeus Inc., Orangeburg, SC). The syringe needle was slowly lowered into the brain to the appropriate stereotaxic coordinates (see specific chapters) using a microdrive apparatus (MO-10; Narishige International, East Meadow, NY). Solution delivery began 5 minutes after lowering to the appropriate depth. Solution delivery was controlled using a 25-µL Hamilton syringe mounted on a KD Scientific model infusion pump (model 100, KD Scientific Inc., Holliston, MA). The needle remained in the brain after completing solution delivery and was slowly retracted after 10 minutes. The overlying burr hole was covered with bone wax and the incision was closed using dissolvable sutures (4-0 Caprosyn™; Covidien; Norwalk, CT). Oral analgesic (RIMADYL® (carprofen), 2 mg p.o.; Bio-Serv; Frenchtown, NJ) was provided following surgical procedures in rats. Topical analgesic ointment (Neosporin® with pramoxine HCl; Rite Aid Corp.) was applied to the incision site in mice following surgical procedures. During surgical procedures and the immediate recovery period following surgery, animals were rested on a heating pad connected to a re-circulating water bath (Gaymar Industries, Inc., Orchard Park, NY) maintained at 37°C. Animals recovered in their home cage under observation in the laboratory (~2 hours) before transport to the animal housing facility. Animal health was assessed daily for a

minimum of 1 week for signs of postoperative distress**.** Particular treatment considerations are explained in greater detail in the subsequent chapters.

In Vivo Electrochemical Recording and Microdialysis – Non-Survival Surgical Procedures

Animals were anesthetized with urethane (1.25 g/kg, 0.9% NaCl i.p.), positioned in a stereotaxic frame designed for the rat or mouse (Kopf Instruments, Tujunga, CA, USA) and rested on a heating pad connected to a re-circulating water bath (Gaymar Industries, Inc., Orchard Park, NY) maintained at 37°C. For electrochemical recordings, a craniotomy was performed for access to the striatum and the overlying dura was reflected prior to insertion of the carbon fiber microelectrode/micropipette assembly (see Electrode Construction and Preparation and Micropipette Preparation). The microelectrode assembly was stereotaxically placed in contact with the brain and lowered to the targeted recording depth(s) using a microdrive apparatus (MO-10; Narishige International, East Meadow, NY). For electrochemical recordings, a burr hole was created (distant from the recording sites) to allow placement of a miniature Ag/AgCl reference electrode (see Preparation of Miniature Reference Electrode) in contact with the brain (Hascup*, et al.*, 2007).

Microdialysis probes were stereotaxically placed in contact with dura and slowly lowered to the desired depth using the vertical arm of the stereotaxic frame.

In vivo Electrochemistry

Principles of Amperometry

By applying a potential to an electrode, the resulting change in current generated by redox reactions involving electroactive species is measured at the electrode surface. Applied potential is maintained by a potentiostat, which maintains a constant potential difference between the recording (working) electrode and the reference electrode half-cell. Current generated from the oxidation and reduction of electroactive molecules is faradaic and linearly correlated with the concentration of electroactive molecules, which donate or accept electrons in established stoichiometric ratios. Although several variations of amperometric measures exist, generally, these techniques provide sensitive measurements and allow for rapid sampling rates (seconds) to monitor neurochemical

events near real-time. For a more detailed review and discussion of electrochemical methods see (Adams, 1990; Cahill*, et al.*, 1996; Gerhardt*, et al.*, 2000).

In our recordings, the Fast Analytical Sensing Technology-16 mark II system (FAST-16 mk II; Quanteon LLC, Nicholasville, KY) was used to amplify, integrate and digitize current measured using carbon fiber microelectrodes (see Electrode Construction and Preparation) and chronoamperometry (see Chronoamperometry).

Chronoamperometry

A square-wave pulse (200 millisecond (msec) duration, 0.0 V (100 msec) to +0.55 V (100 msec)) (Figure 2.1) was applied to the carbon fiber microelectrode *versus* a Ag/AgCl reference electrode and repeated at 1 Hz (FAST-16 mk II system; Quanteon LLC, Nicholasville, KY). The analyte of interest (DA in this example) was oxidized at +0.55 V, producing an increase in current detected by the electrode. The oxidation product (dopamine-*o*-quinone) was reduced when the potential was stepped back to 0.0 V, also producing a change in current measured at the electrode surface (Figure 2.1). The reduction:oxidation ratio (Figure 2.2) is a distinguishing feature for some electroactive species in the brain and is used to confirm chemical identity (Gerhardt and Hoffman, 2001). Oxidation and reduction currents were digitized during the last 80 ms of each pulse using the FAST-16 mkII recording system (Quanteon, LLC, Nicholasville, Kentucky) as outlined in previous work from our laboratory (Gerhardt*, et al.*, 1986; Hebert*, et al.*, 1996).

Electrode Construction and Preparation

Carbon fiber microelectrodes were manufactured using minor modifications from previously established methods (Gerhardt and Hoffman, 2001). Briefly, single carbon monofilament (30-33 µm diameter “fibers”)(Specialty Materials Inc., Lowell, Massachusetts) was threaded into a pulled (Kopf Instruments, Tujunga, CA) glass capillary (Sodalime; 4 mm O.D., 0.7 mm wall thickness (before pulling); Glass Warehouse, Millville, NJ) and secured using Epoxylite® (#6001; Electrical Insulation Suppliers (EIS), Atlanta, GA). The capillary tube was filled with graphite-epoxy resin (PX grade; Dylon Industries Inc., Cleveland, OH), excess glass was trimmed and a 30-gauge enamel-coated copper wire (#278-1345; Radio Shack) was inserted into the graphite-epoxy and secured with Epoxylite®. The pulled end of the glass capillary was bumped and the exposed carbon-fiber tip was trimmed to a length of ~150 µm.

The sensitivity and selectivity for DA *versus* other endogenous electroactive molecules were enhanced by coating the electrode tips with Nafion® (5% solution, Aldrich Chemical Co., Milwaukee, WI) (Gerhardt and Hoffman, 2001). Nafion® is a perfluorosulfonated Teflon® derivative that is permeable to cationic species but not anionic species. Thus, potential interfering currents generated by the oxidation/reduction of acidic electroactive compounds in the brain were minimized (Gerhardt*, et al.*, 1984). Carbon fiber electrodes were baked at 200°C for 5 minutes and then slowly swirled (~4 times, 4 seconds total) in Nafion® solution. Electrodes were baked again for 5 minutes at 200°C. For connection to the FAST-16 mark II headstage/potentiostat (Quanteon, LLC, Nicholasville, KY), a gold pin amphenol (Mill-Max Mfg. Corp., Oyster Bay, NY; Part no: 3603-0-07-21-00-00-08-0) was soldered to the copper wire end of each carbon fiber electrode.

Electrode Calibration

Nafion®-coated carbon fiber electrodes were calibrated prior to use *in vivo.* Phosphate buffered saline (PBS) solution (0.05 M, pH = 7.4) was gently stirred using a battery-operated, portable magnetic stir plate (Barnant Co., Model #700-0153). The glass Ag/AgCl reference electrode (Bioanalytical Systems, West Lafayette, IN; Model #RE-5B) and recording tip of the Nafion®-coated carbon fiber microelectrode were submerged in the buffer solution. High-speed *in vivo* chronoamperometric measurements were carried out as previously described (Chronoamperometry). Parameters quantified included: linearity of response to DA, selectivity and limit of detection (LOD) for DA (Figure 2.3). Microelectrodes showed linear responses to serial additions of DA (2 – 6 μM, effective beaker concentrations). Additions of ascorbate (250 μM, Fisher Scientific) were used to determine the selectivity of the recordings for DA. Changes in DA concentration *in vivo* were expressed as change from a stable baseline using the calibration curve for each particular microelectrode. Specific calibration values are described in the methods sections of the subsequent chapters.

Preparation of Miniature Reference Electrode

A miniature Ag/AgCl reference electrode (A-M Systems, Inc., Catalog #786500; 0.008 inch bare, 0.011 inch coated) was used for *in vivo* electrochemical recordings. Reference electrodes were prepared by stripping the Teflon® coating from the tip (~1 cm) of each end of the silver wire. One end was soldered to a gold pin amphenol (Mill-

Max Mfg. Corp., Part #3603). The other end was submerged in a 1M HCl solution that was saturated with NaCl. Using a 9-volt power supply (Elenco Precision, Model XP-620), the reference electrode was anodized while submerged in the plating bath solution. A platinum wire connected to the negative lead (the cathode) was also placed in the plating solution. The potential was applied for approximately 10 minutes and reference electrodes were stored in 3M NaCl for at least 1 hour prior to use *in vivo*.

Micropipette Preparation

For pharmacological manipulation *in vivo,* a glass micropipette (A-M Systems, Inc., Everett, WA; 1 mm o.d., 0.58 i.d.,) was pulled using a vertical pipette puller (Kopf Instruments, Tujunga, CA) and bumped to an inner tip diameter of 7-15 µm. The micropipette was then affixed flush with the Nafion®-coated microelectrode (Figure 2.1) at a distance of ~200 µm using sticky wax (Kerr Corp., Orange, CA, USA).

Local Application of Solutions *In Vivo*

Physiological solutions were filtered (0.22 µm) and pH adjusted (7.2-7.4) before filling the micropipette for *in vivo* application. After stereotaxic placement of the microelectrode/"filled"-micropipette assembly into the brain, baseline recordings were carried out for a minimum of 1 hour. When a stable baseline was reached, the recording assembly was lowered to the targeted recording depth. Local application of solutions was accomplished by pressure ejection of nitrogen gas (Palmer*, et al.*, 1980). A picosprizter III (Parker-Hannifin, NJ) was used to control the amount of solution applied by manipulating the pressure (10-30 psi) or duration (0.5-2.5 seconds). In order to determine the amount of solution ejected, the solution volume (displacement) was monitored using a stereomicroscope fitted with a reticule (Friedemann and Gerhardt, 1992). The purpose and constituents of solutions used are addressed in the subsequent chapters.

Tissue Preparation and Neurochemical Analysis of Tissues (HPLC-EC)

All tissue samples to be analyzed for neurochemical content were placed into pre-weighed microcentrifuge tubes, weighed and placed on dry ice. After dissections, tubes containing tissues were stored at -80°C until analysis by high-performance liquid chromatography with electrochemical detection (HPLC-EC). Technical details about dissection methods are explained in the subsequent chapters.

In preparation for HPLC-EC analysis, samples were sonicated in cold mobile phase (pH = 4.05-4.15) with dihydroxybenzylamine (DHBA) as an internal standard. Samples were centrifuged at 16,000 × g for 10 minutes (-4 °C). The filtered supernatants were injected (50 µL) into the HPLC system equipped with dual-coulometric electrochemical detectors (Hall*, et al.*, 1989) (also see Neurochemical Determination by HPLC-EC). The system detection limits were < 1 ng/g tissue wet weight for the analytes of interest. Dopamine and metabolites of DA (DOPAC and HVA) were identified by peak retention time (determined from standards) and were quantified by peak area using standard concentrations of analytes. Neurochemical content was expressed as ng/g wet weight of tissue. Neurochemical analyses of tissues using HPLC-EC were performed by Stewart Surgener (Chapter 4) and Wayne Cass, Ph.D. (Chapter 3, Chapter 5).

Portions of these methods have been previously published in the manuscript:

Littrell, O.M., Pomerleau, F., Huettl, P., Surgener, S., McGinty, J.F., Middaugh, L.D., Granholm, A.C., Gerhardt, G.A., Boger, H.A. 2010. Enhanced dopamine transporter activity in middle-aged Gdnf heterozygous mice. Neurobiol Aging.doi:S0197-4580(10)00446-X[pii] 10.1016/j.neurobiolaging.2010.10.013.

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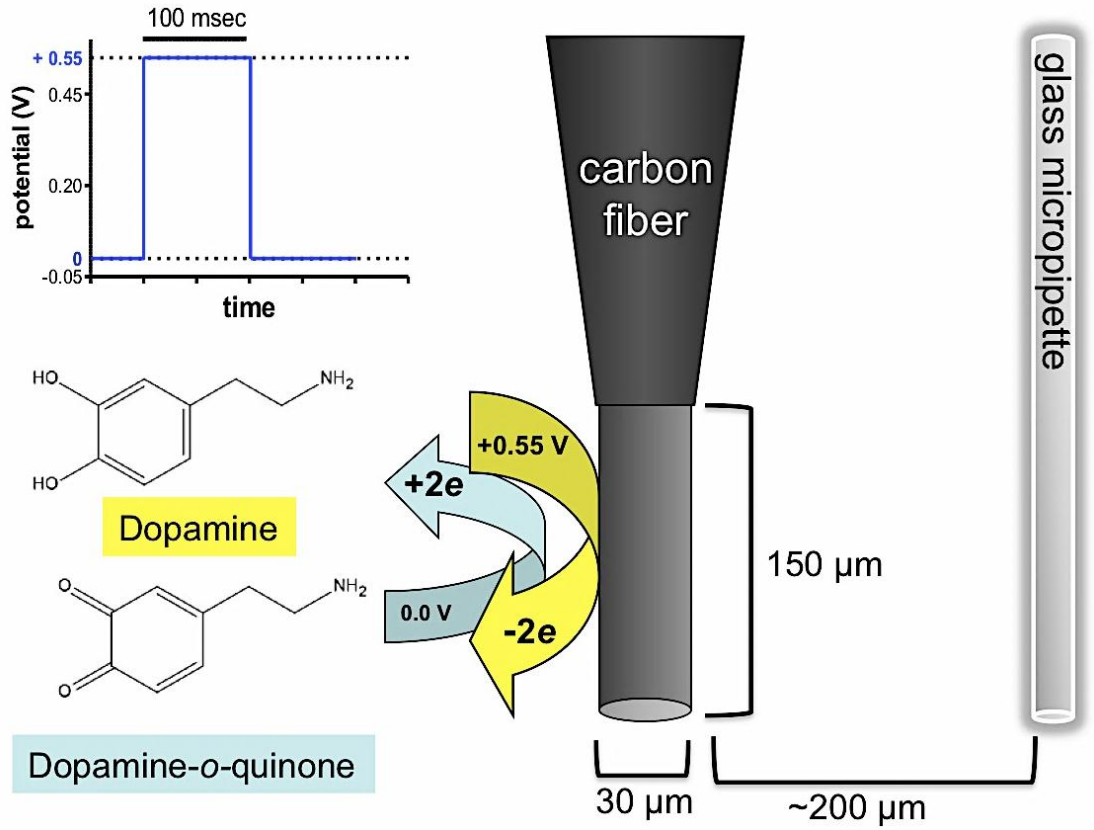

Figure 2.1 Chronoamperometry with Carbon-Fiber Microelectrodes: Recording Assembly used for Electrochemical Detection of Dopamine
Inset: A square wave potential is applied to the working electrode *versus* a Ag/AgCl reference electrode. Square wave potentials (+0.55 V (100 ms) and 0.0 V (100 ms)) were repeated at 1 Hz (inset). The resulting oxidation and reduction currents were digitally integrated during the last 80 ms of each pulse and averaged to a 1 Hz sampling rate. In addition to high temporal resolution (seconds), electrochemical techniques using microelectrodes provide a high degree of spatial resolution (microns) because only molecules that come in contact with the recording electrode surface are detected. A micropipette can be affixed flush with the microelectrode in order to locally apply solutions.

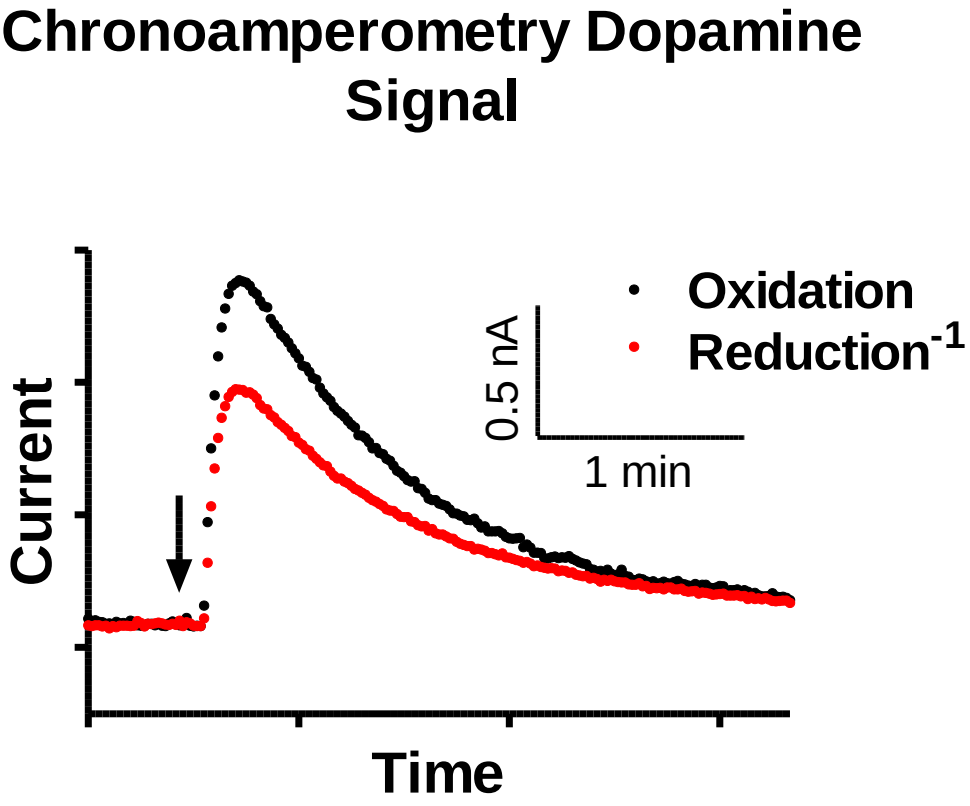

Figure 2.2 Chronoamperometric Measures for the Detection of Dopamine
Local application of DA in the brain (arrow) results in a transient change in current measured by the electrode (inset: axes units). Dopamine signals may be differentiated from other electroactive species based on the ratio of reduction:oxidation currents (Gerhardt and Hoffman, 2001).

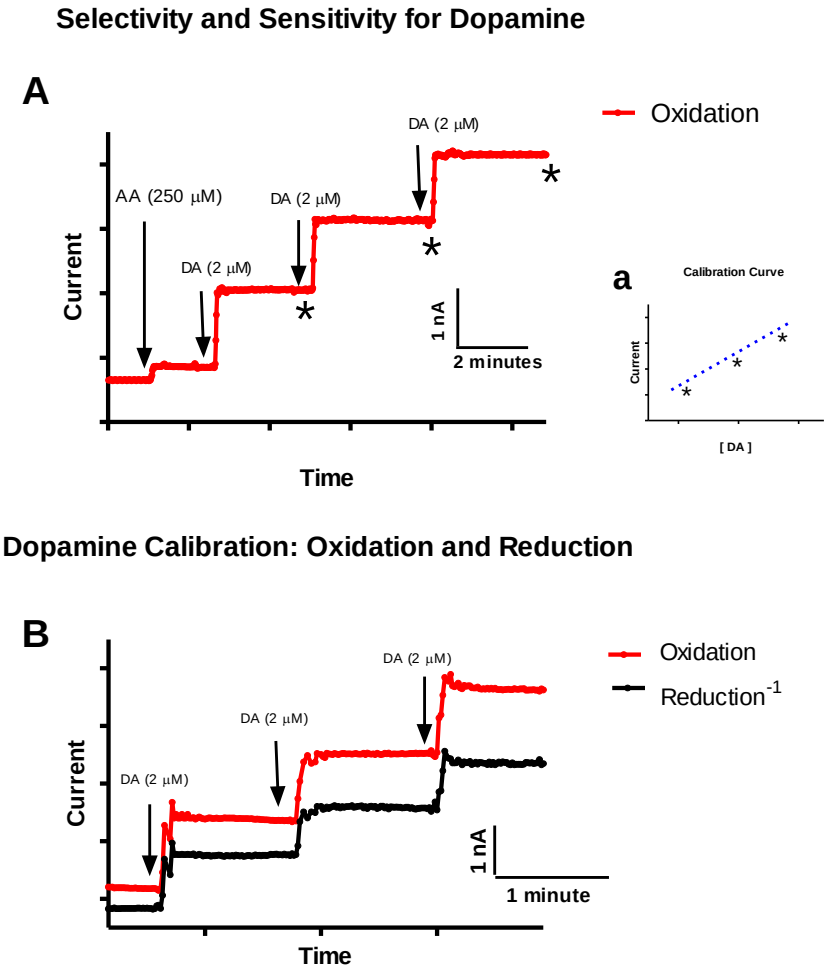

Figure 2.3 *In vitro* Carbon Fiber Microelectrode Calibration
A) Oxidation current is shown for a carbon fiber microelectrode calibrated *in vitro* for selectivity and sensitivity for DA (inset: axes units). Nafion®-coated microelectrodes are challenged with an addition of ascorbate (AA), an electroactive molecule present in high concentration in the brain. Three serial additions of DA demonstrate a comparatively robust increase in current that is linear ($R^2 > 0.99$) with respect to concentration. Inset: (a) The change in oxidation current is used to calculate the sensitivity (slope, nA/μM) of individual electrodes in order to correlate oxidation current change with a DA-concentration value. The limit of detection (LOD, μM) is calculated by dividing the standard deviation of the baseline oxidation current (nA) by the slope (nA/μM) using a

signal:noise ratio of 3. B) Reduction and oxidation currents following sequential additions of DA are shown (inset: axes units).

Chapter Three: Characterization of the Neurochemical Effects of DNSP-11

Introduction

Parkinson's disease is characterized by the loss of DA neurons in the SN and a concomitant decrease in synaptic DA in the striatum (Bernheimer*, et al.*, 1973). Current therapies may improve motor deficits associated with nigrostriatal dysfunction by enhancing DA-synthesis and/or decreasing -metabolism (Goudreau, 2006; Salawu*, et al.*, 2010). Unfortunately current therapies are only symptomatic treatment regimens, which fail to slow the disease progression and often cause unfavorable side effects (Muller and Russ, 2006; Salawu*, et al.*, 2010). One promising PD therapeutic agent, GDNF, has shown robust restorative and protective effects on DA neurons (Grondin and Gash, 1998; Hebert and Gerhardt, 1997; Kearns*, et al.*, 1997; Lin*, et al.*, 1993). Despite evidence for therapeutic potential from phase I clinical trials (Gill*, et al.*, 2003; Slevin*, et al.*, 2005), the clinical utility of GDNF has been questioned because of evidence for toxicity (Lang*, et al.*, 2005), limited diffusion to target areas (Salvatore*, et al.*, 2006; Sherer*, et al.*, 2006) and phase II trials that did not reach proposed endpoints. Considering the potential of neurotrophic factors as therapeutics in neurodegenerative disease (Peterson and Nutt, 2008) and particular delivery issues related to binding characteristics of GDNF (Salvatore*, et al.*, 2006), there has been a recent interest in small peptides with neurotrophic-like properties that may offer therapeutic benefit and avoid delivery limitations (Bradley*, et al.*, 2010; Kelps*, et al.*, 2011).

GDNF and other neurotrophic factors are processed as immature precursor proteins with a pro-region – absent from the mature secreted GDNF protein (Chao and Bothwell, 2002). The pro-region is predicted to yield small amidated peptides that have been investigated by our group and independently by others (Figure 3.1) (Bradley*, et al.*, 2010; Immonen*, et al.*, 2008; Kelps*, et al.*, 2011). Peptides from the pro-region of neurotrophic factors (pro-peptides) have been suggested to regulate folding and secretion of the mature secreted protein (Gray and Mason, 1990), however, evidence for sequence conservation among related species suggest potential for distinct biological activity of pro-peptides (Immonen*, et al.*, 2008).

An 11-amino-acid sequence derived from the GDNF pro-sequence in the rat (Immonen*, et al.*, 2008) and human (Bradley*, et al.*, 2010; Kelps*, et al.*, 2011) (Table 3.1) has demonstrated biological effects including: 1) enhanced survival and morphological complexity of primary DA-neuron cell cultures 2) protection from cytotoxicity of 6-

hydroxydopamine, staurosporine and gramicidin 3) increased resting extracellular levels of DA *in vivo* (Bradley*, et al.*, 2010) and 4) increased excitability of rat CA1 pyramidal neurons (Immonen*, et al.*, 2008). In the described studies, we further investigated *in vivo* biological activity of pro-peptides with particular attention to neurotrophic-like effects in the nigrostriatal pathway. DNSP-11 was further investigated for enhancing effects in the nigrostriatal pathway of the F344 rat with the general hypothesis that DNSP-11 enhances dynamic properties of DA-neuron function in the nigrostriatal pathway. A single treatment of DNSP-11 was administered to the primary cell bodies of the SN and *in vivo* electrochemistry was used to measure DA release from discrete striatal sub-regions at designated time points: 1-, 2-, and 4-weeks after treatment. Thus, these studies determined if there were heterogeneous effects of DNSP-11 on striatal sub-regions and when the peak effect of DNSP-11 occurred. Additionally, neurochemical content of striatal and nigral tissues was investigated to determine if there were indications for DA-synthesis or -metabolism modulation after DNSP-11 treatment.

The post-translational processing model of GDNF predicts the generation of an additional 5-amino-acid peptide (Figure 3.1; Table 3.1) termed dopamine-neuron stimulating peptide-5 (DNSP-5), which has also been examined for biological activity (Bradley*, et al.*, 2010; Immonen*, et al.*, 2008; Kelps*, et al.*, 2011). DNSP-5 has been shown to: 1) increase morphological features of DA neurons in cell culture 2) protect cells from staurosporine-induced cytotoxicity and 3) increase extracellular levels of DA *in vivo* (Richardson, 2009; Fuqua, 2010). Because DNSP-11 and DNSP-5 are predicted to be generated concurrently, both peptides were delivered to the SN and striatal microdialysis was performed to determine if the peptide mixture enhanced DA-neuron function – as demonstrated for a single treatment of DNSP-11 or DNSP-5 (Bradley*, et al.*, 2010; Fuqua, 2010). Striatal and nigral tissues were also collected to determine if there were changes in DA-synthesis or -metabolism after DNSP-5/-11 treatment.

Materials and Methods

Reagents

DNSP-11 peptides were synthesized and purified by W.M. Keck Foundation (Yale University, New Haven, CT). DNSP-5 peptides and control peptides were synthesized and purified to >98% using reverse phase high-pressure liquid chromatography by AC Scientific (Duluth, GA). The control peptide (scrambled 11mer)

consisted of identical amino-acid constituents but different sequence than DNSP-11 (Leu-Pro-Ser-Pro-Arg-Glu-Asp-Ala-Glu-Pro-Ala-amide).

Delivery of DNSPs

Peptides were dissolved (4 mM) in sterile (0.22 µM filtered) citrate buffer (10 mM sodium citrate, 150 mM NaCl, pH = 5) (Bradley, et al., 2010). Peptide and citrate buffer (vehicle) aliquots were stored at (-80°C). Animals (see Rat Studies) were treated using 2 infusion points just dorsal to the right SN as described in previous studies using GDNF (Hebert*, et al.*, 1996). A single cranial burr hole was adequate to access both infusion points. Stereotaxic coordinates from *bregma* were (mm): Point 1: anterior-posterior: -5.6, medial-lateral: - 1.7, dorsal-ventral: -7.3; Point 2: anterior-posterior: -5.6, medial-lateral: -2.5, dorsal-ventral: -6.8; incisor bar: -2.3; (Paxinos and Watson, 2005). See also Intracranial Drug Delivery - Survival Surgical Procedures.

For electrochemical studies, animals were treated with: 1) DNSP-11 (30 µg), 2) control peptide (30 µg) or 3) citrate buffer vehicle (equivalent volume) (n = 7-9). A total of 5 µL (0.25 µL/min) of solution was delivered to the SN (divided equally between the two points).

In the microdialysis studies either 1) DNSP-11 (10 µg) + DNSP-5 (10 µg) (equal mass solution) or 2) citrate buffer (vehicle) (equivalent volume) was administered (n = 4) using the delivery methods described (Intracranial Drug Delivery - Survival Surgical Procedures).

Electrochemical Recordings (DNSP-11)

Animals and materials were prepared for electrochemical recordings (In Vivo Electrochemical Recording and Microdialysis – Non-Survival Surgical Procedures; In vivo Electrochemistry) at the designated time points after unilateral DNSP-11, vehicle or control treatment. The glass micropipette was filled with an isotonic potassium chloride solution (120 mM KCl, 29 mM NaCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, pH =7.2-7.4) used to locally depolarize striatal terminals (Gerhardt*, et al.*, 1986). The carbon fiber/micropipette assembly was stereotaxically placed and lowered into the striatum. Stereotaxic coordinates used were (from *bregma* (mm)): Site 1: anterior-posterior: 0, medial-lateral: -2.3, dorsal-ventral: -3.5 to -7.5; Site 2: anterior-posterior: +1.0, medial-lateral: -2.3, dorsal-ventral: -3.5 to -7.5; incisor bar: -2.3 (Paxinos and Watson, 2005) (Figure 3.2).

After reaching a stable baseline (~1 hour of baseline recording following initial placement in the brain), the recording assembly was lowered along the dorsal-ventral axis of the striatum. The potassium solution was locally applied (75-125 nL) (see Local Application of Solutions In Vivo) to elicit a single DA signal from each recording depth (500 µm increments; dorsal-ventral: -3.5 to -7.5) (Figure 3.3). When the DA signal had returned to a stable baseline, the recording assembly was lowered 500 µm to the next recording depth. Baseline recordings were obtained for a minimum of 5 minutes before local application of potassium solution at the next recording depth. Because these data demonstrated greater variability within an individual animal (between recording depths) than between animals (at the same recording depth), each recording point (depth) was treated as an individual sample (*n*) as previously outlined (Cass and Gerhardt, 1994; Hebert*, et al.*, 1996). Based on the heterogeneity of the origin of DA terminals in the striatum (Beckstead*, et al.*, 1979) and appreciable variability in amplitude among dorsal and ventral recording depths (Figure 3.3), data were grouped and analyzed by striatal sub-region: dorsal, intermediate and ventral. Each sub-region included DA-release data from three depths: -3.5, 4.0, -4.5 (dorsal), -5.0, -5.5, -6.0 (intermediate), -6.5, -7.0, -7.5 (ventral).

High–speed chronoamperometric data were analyzed using custom MATLAB®-based software (The MathWorks™, Inc.). The DA signal amplitudes were quantified as a transient and robust increase/change (Δ) from the baseline oxidation current (nA). The change was then divided by the electrode slope (nA/µM) to calculate the concentration change in DA (µM) (Hebert*, et al.*, 1996) (Figure 2.3; Figure 3.3). All signals used for analysis exhibited reduction:oxidation ratios of 0.74 ± 0.003 (mean ± SEM), n = 1014 (signals) consistent with the established profile for DA (Gerhardt and Hoffman, 2001). Microelectrodes used in the described experiments were selective for DA (≥ 99:1, *versus* ascorbate), exhibited linear responses to DA (R^2 ≥ 0.97) and demonstrated an LOD of 39 ± 6 (nM) using a signal:noise ratio ≥ 3 (mean ± SEM, n = 68, microelectrodes).

Histology

After electrochemical recordings, animals were decapitated while under deep anesthesia and the brains were dissected and frozen until preparation to cryo-section (40 µm; Microm, Zeiss, and Thornwood, NY, USA). Sections were mounted on glass slides, stained with cresyl violet acetate and cover-slipped. Placement of

microelectrodes within the striatum was examined in histological sections. The observed recording tracts are shown (Figure 3.2).

Determination of Tissue Neurochemical Content by HPLC-EC

In a separate cohort of animals, neurochemical content of striatal and nigral tissues were determined for DNSP-11 and vehicle treated animals (4 weeks after treatment, *n* = 6-8). Tissues from animals used in microdialysis studies (DNSP-5/-11 and vehicle) (see Delivery of DNSPs) were also collected for neurochemical analysis. Animals were euthanized via CO_2 asphyxiation and decapitated. Brains were placed in an ice-cold rat brain matrix and sectioned into 1 mm slices. Based on heterogeneous DA innervation of the striatum (Swanson, 1982), the treated striata were dissected into dorsal and ventral sub-regions. The treated SN was also dissected. Samples from the treated striata and SN were collected and analyzed (see Tissue Preparation and Neurochemical Analysis of Tissues (HPLC-EC)). Analyses of tissue by HPLC-EC were performed by Wayne Cass, Ph.D.

Microdialysis

Microdialysis probes (CMA 11, CMA Microdialysis, Stockholm, Sweden; 4 mm membrane length) were prepared by perfusion with 70% ethanol (3 µL/min) followed by water (~0.2 µL/min) until probe recovery determination. Before *in vivo* microdialysis, probes were calibrated for percent recovery *in vitro.* Microdialysis probes were submerged in a standard solution of analytes of interest:

100 nM 3,4-dihydroxyphenylacetic acid (DOPAC)

200 nM dopamine (DA)

100 nM homovanillic acid (HVA)

Probes were perfused with physiological artificial cerebrospinal fluid (aCSF) (1 µL/min) for 20 minutes to collect the probe recovery sample from the standard solution. Percent recovery for each microdialysis probe was determined for individual analytes and used to normalize microdialysis data. Microdialysis probe percent recovery ranged from 22-33% (for DA).

Gastight® syringes (Hamilton USA, Reno, NV; 1000 µL) containing aCSF solutions were connected to microdialysis probes using Teflon® tubing (FEP tubing, 0.12 mm inner diameter) and tubing adapters (CMA Microdialysis, Stockholm, Sweden). A KD scientific syringe pump (KDS230, Holliston, MA) was used to control perfusion rate (1

µL/min). Syringes were connected to a liquid switch (CMA/110, CMA Microdialysis, Stockholm, Sweden) to alternate aCSF solutions*:

1) physiological aCSF (124 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$•2 H_2O, 1 mM $MgCl_2$•6 H_2O, 1 mM NaH_2PO_4•H_2O, 25 mM $NaHCO_3$, 5.9 mM d-glucose)
2) high-potassium aCSF (27 mM NaCl, 100 mM KCl, 1 mM $CaCl_2$•2 H_2O, 1 mM $MgCl_2$•6H_2O, 1 mM NaH_2PO_4•H_2O, 25 mM $NaHCO_3$, 5.9 mM d-glucose)
(3) d-amphetamine aCSF (250 µM d-amp (Sigma-Aldrich), 124 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$•2 H_2O, 1 mM $MgCl_2$•6 H_2O, 1 mM NaH_2PO_4•H_2O, 25 mM $NaHCO_3$, 5.9 mM d-glucose) (pH adjusted to 7.2-7.4)

*Reagents for aCSF solutions were purchased from Fisher Scientific

All aCSF solutions were gassed (95% O_2, 5% CO_2) and contained 500 µM ascorbate.

Microdialysis probes were stereotaxically placed in the right striatum (see In Vivo Electrochemical Recording and Microdialysis – Non-Survival Surgical Procedures) as described for previous studies using DNSP-11 (Bradley*, et al.*, 2010). Stereotaxic coordinates were (from *bregma* (mm)): anterior-posterior: +1.5; medial-lateral: -2.3; dorsal-ventral: -8.0; incisor bar: -2.3 (Paxinos and Watson, 2005). Artificial cerebrospinal fluid was perfused through the probes at a rate of 1 µL/min and dialysate samples were collected at 20-minute intervals (Figure 3.8).

Baseline neurochemical levels were determined from dialysates collected during perfusion of isotonic (1) aCSF (samples 3-5). Evoked DA release was measured during perfusion of: (2) high-potassium aCSF (sample 7) and (3) d-amphetamine aCSF (sample 12). Dialysate samples were analyzed by HPLC-EC (see Neurochemical Determination by HPLC-EC) for DA and its primary metabolites: DOPAC and HVA.

Neurochemical Determination by HPLC-EC (Microdialysis Dialysates)

Quantification of analytes in dialysate samples was done using HPLC-EC (Beckman, Inc., Fullerton, CA) methods modified from an established protocol for the detection of catecholamines (Hall, et al., 1989). Briefly, a citrate-acetate buffer mobile phase was used (4% methanol, 0.34 mM 1-octane-sulfonic acid, pH = 4.05-4.15) at a flow rate of 1.7 mL/min. Analytes were separated with reverse phase chromatography using a C18 column (4.6 mm x 75 mm, 3 µm particle size, Shiseido CapCell Pak UG120,

Shiseido Co., LTD., Tokyo, Japan). Dual-channel coulometric detectors were used for electrochemical detection (ESA model 5014B dual analytical cell; E1 = +300 mV; E2 = -250 mV, pre-conditioning cell = +100 mV). Standards (see Microdialysis) were used to determine retention time and concentration based on peak area.

Data Analysis and Statistics

Values > 2 standard deviations from the mean were removed from all data. Statistical significance was defined as $p < 0.05$ for all analyses. In reported statistics, *n* represents the number of animals unless indicated otherwise.

Results

DNSP-11 Effects on Dopamine Release (*In vivo* Electrochemical Studies)

Electrochemical methods were used to investigate sub-regional effects on potassium-evoked DA release within the dorsal-ventral orientation of the striatum. These studies also examined the duration of effects from a single treatment because experiments were performed 1-, 2- and 4-weeks after a single unilateral treatment (DNSP-11, vehicle or control peptide) to the SN. Vesicular DA release was stimulated by local application of a high-potassium solution and the subsequent release was measured by high-speed chronoamperometry. Measurements were taken every 500 µm to obtain a striatal depth profile for DA release at each time point. Depth-profile analysis of the striatum was conducted comparing DNSP-11 and vehicle treatment for several time points: 1 week (Figure 3.4), 2 weeks (Figure 3.6), and 4 weeks (Figure 3.7) after treatment. Control peptide treatment was examined only at the 2-week time point due to peptide availability.

For all time points, an appreciable decline in amplitude was apparent in more ventral recording depths compared to dorsal recording regions (Figures 3.4, 3.6, 3.7; statistical analysis not shown). This decline is consistent with previous studies using the same methodology (Gerhardt*, et al.*, 1986; Hebert*, et al.*, 1996) and reflects a corresponding decrease in DA nerve terminal density in the ventral striatum (Oke*, et al.*, 1978). Of greater interest than the depth variable was the effect of DNSP-11 treatment. The striatal depth profiles and sub-regional analysis 1- (Figure 3.4) and 4-weeks (Figure 3.7) after treatment did not indicate changes in release amplitude for any recording depth or striatal sub-region ($p > 0.05$, all). DNSP-11 treatment did, however, demonstrate increased DA release 2 weeks after treatment (Figures 3.5, 3.6). A control

peptide (scrambled 11mer), which became available during these studies, was added as an additional treatment group to the 2-week studies (Figure 3.5, 3.6). This group was included to investigate the potential effects of a control peptide on DA release. DNSP-11 treatment increased DA-release amplitude ($F_{2,185}$= 12.88, $p < 0.0001$), which occurred at two individual recording depths in the rostral recording site (AP +1.0) *versus* the control peptide (Figure 3.6 A): [mean ± SEM (μM); Dorsal-Ventral: 4.0 mm $^{**}p < 0.01$, Control: 2.13 ± 0.40; DNSP-11: 4.26 ± 0.84; Dorsal-Ventral: 4.5 mm, $^{**}p < 0.01$ Control: 2.11 ± 0.30; DNSP-11: 4.46 ± 0.62; $n = 8$]. There were no significant effects of treatment on DA-release amplitude ($p > 0.05$) for any individual recording depth in the caudal (AP 0) recording site. Sub-regional analysis of the striatum (Figure 3.6 C, D) shows a significant effect of treatment [AP +1.0: $F_{2,203} = 13.04$, $p < 0.0001$, AP 0: $F_{2,192}= 3.13$, $p = 0.0462$] on amplitude at both recording sites. DNSP-11 increased DA release in the dorsal striatum [mean ± SEM (μM) AP +1.0: vehicle: 2.77 ± 0.28, $n = 23$; control: 2.11 ± 0.18, $n = 24$; DNSP-11: 4.00 ± 0.44 $n = 23$, $^{**}p < 0.01$ *versus* vehicle; $^{***}p < 0.001$ *versus* control; AP 0: vehicle: 3.47 ± 0.36, $n = 23$; control: 3.46 ± 0.31, $n = 24$; DNSP-11: 4.64 ± 0.58, $n = 24$, $^{*}p < 0.05$ *versus* vehicle; $^{*}p < 0.05$ *versus* control; *n* represents the number of signals] and intermediate striatal sub-regions [mean ± SEM (μM) AP +1.0 control: 1.99 ± 0.20, $n = 24$; DNSP-11 3.02 ± 0.34, $n = 24$ $^{*}p < 0.05$ *versus* control; *n* represents the number of signals].

In summary, the greatest effect of DNSP-11 occurred 2 weeks after treatment and exhibited selectivity for the dorsal and intermediate regions of the striatum. Additionally, there were no differences ($p > 0.05$) in DA-release amplitude between control peptide and vehicle treatment groups.

DNSP-11 Effects on Tissue Neurochemical Content

To evaluate if DA-synthesis, -storage or -metabolism were affected by DNSP-11, striatal and nigral tissues were collected and analyzed by HPLC-EC for neurochemical content. Dopamine, primary metabolites (DOPAC, HVA) and turnover ratios were unchanged ($p > 0.05$, all) 4 weeks after DNSP-11 treatment (Table 3.2).

DNSP-5/DNSP-11 Effects on Basal Neurochemical Levels (*In vivo* Microdialysis)

Because DNSP-11 and DNSP-5 have demonstrated increased basal concentrations of DA and DA metabolites (Bradley*, et al.*, 2010; Fuqua 2010), *in vivo*

microdialysis of the striatum was used to determine if co-injection of DNSP-5 and DNSP-11 also enhanced DA-neuron function. Extracellular basal concentrations of DA, DOPAC and HVA were not changed after DNSP-5/DNSP-11 treatment ($p > 0.05$, all). Evoked DA release by d-amphetamine and potassium were not significantly changed ($p > 0.05$, both) by DNSP-5/DNSP-11 (Table 3.3).

DNSP-5/DNSP-11 Effects on Tissue Neurochemical Content

To evaluate if DA-synthesis, -storage or -metabolism were affected by co-injection of DNSP-5/-11, striatal and nigral tissues were collected from DNSP-5/DNSP-11 treated animals and analyzed by HPLC-EC for neurochemical content (Table 3.4). There were no changes in DA or DOPAC between vehicle and DNSP-5/DNSP-11 treated striata (dorsal and ventral sub-regions), however, HVA levels were significantly increased by DNSP-5/DNSP-11 treatment (*p = 0.0362, (dorsal striatum only). A significant change was seen in the SN after DNSP-5/-11 treatment with increased tissue levels of DA (*p = 0.0331), DOPAC (*p = 0.0015*)* and HVA (*p = 0.0270).

Turnover ratios were not changed ($p > 0.05$) in the striatum or SN by DNSP-5/DNSP-11.

Discussion

The major finding of these studies is that DNSP-11 treatment increased evoked DA release. An increase in depolarization-induced DA release may indicate modulation of several aspects of DA release: DA transporter function, number and/or size of DA vesicles, vesicular docking and fusion modulation resulting in altered equilibrium between vesicular release and cytoplasmic DA concentrations and/or ion-channel modulation of the depolarization event used to stimulate release (Bark and Wilson, 1994; Gerhardt*, et al.*, 1986; Gillis*, et al.*, 1996; Rosenmund and Stevens, 1996; Shui*, et al.*, 1998). Although determining the specific underlying mechanisms of the enhancing effects of DNSP-11 on DA release is beyond the scope of these studies, increased DA synthesis/storage is unlikely because neurochemical content of tissues did not indicate changes in DA or DA metabolites. The electrochemical studies highlight important similarities between DNSP-11 and GDNF, which both demonstrate increased evoked DA release in the rat (Hebert*, et al.*, 1996) without a parallel increase in tissue neurochemical content of DA (Hebert*, et al.*, 1996). Both GDNF and DNSP-11 appear to enhance DA-neuron function through a presynaptic release mechanism, however, the

difference in the durational profile and spatial presentation of effects provide distinctions between GDNF and DNSP-11.

Interestingly, co-injection of 2 pro-peptides (DNSP-5/DNSP-11), predicted to be generated concurrently, does not affect extracellular DA or DA metabolite levels in the striatum. Analysis of tissue neurochemical content, however, suggests increased DA-synthesis and -metabolism in the SN. The relationship between cellular pathways and neurochemical effects associated with different DNSPs and, potentially, GDNF is an interesting aspect of pro-peptide function to be investigated in future studies.

DNSP-11 Treatment Causes Long-term Changes in Dopamine-Neuron Function

DNSP-11 increased DA release 2 weeks after a single treatment. The time course supports a robust effect on synaptic function that suggests plastic changes in the nigrostriatal pathway (Bezard and Gross, 1998). Long-term changes in synapse structure may contribute to the observed functional changes. Proposed changes in synapse structure are supported by previous work that demonstrated increased morphological differentiation of DA neurons *in vitro* after DNSP-11 treatment (Bradley*, et al.*, 2010). Bradley *et al.,* also reported a marked and rapid decline in immunohistochemical labeling of DNSP-11 (3 hours after treatment) and near undetectable levels by 24 hours. Rapid (hours) clearance of DNSP-11 relative to the presentation of neurochemical effects (weeks) support that DNSP-11 induces long term changes in DA-neuron function. Such rapid clearance of DNSP-11 *in vivo* prompted examination of earlier time points (1- and 2-week). Evidence for increased basal concentrations of DA and DA metabolites (4 weeks after treatment (Bradley*, et al.*, 2010)) prompted examination of evoked release at 4 weeks. Unfortunately, the 3-week time point was not examined in the DNSP-11 studies for direct comparison to GDNF studies. Although the peak effect on DA release by DNSP-11 (2 weeks) is earlier than the peak effect seen with GDNF (3 weeks, Hebert*, et al.*, 1996), DNSP-11 and GDNF exhibit comparable (weeks) long-term effects, which are characteristic of transforming growth factors like GDNF (Hudson*, et al.*, 1995; Lin*, et al.*, 1993). Thus, the general time course of enhanced *in vivo* functional measures after DNSP-11 or GDNF treatment is comparable (weeks). The rapid (hours) clearance of exogenous DNSP-11 (Bradley*, et al.*, 2010) contrasts with studies using GDNF that have shown GDNF protein detection 7 days after a single treatment (Lapchak*, et al.*, 1997b). Furthermore, evidence that

DNSP-11 does not interact with the GFRα1 receptor is another disparity and supports that DNSP-11 and GDNF do not act through the same receptor (Bradley*, et al.*, 2010). Although a receptor has not been determined for DNSP-11, the discrepancy of GFRα1-receptor preference between GDNF and DNSP-11 strongly supports fundamental mechanistic differences. Although the subtle differences in functional measures (time course and sub-regional effects) also support this concept, direct comparison of the electrochemical functional measures is problematic without inclusion of a GDNF treatment group in the current studies. Direct comparison of the functional measures should be considered with caution.

Enhanced Dopamine Release in the Dorsal Striatum Indicates a Selective Effect on the Substantia Nigra

Importantly, these studies indicate that treatment with an 11-amino-acid control peptide, with identical composition but different sequence than DNSP-11, does not affect DA release. Additionally, the control peptide did not exhibit different neurochemical effects *versus* vehicle treatment (citrate buffer only). Electrochemical studies designed to characterize evoked DA release in discrete striatal sub-regions indicated significant increases in evoked DA release in the dorsal striatum of DNSP-11 treated animals (AP +1.0: 44% increase *versus* vehicle, 90% *versus* control peptide; AP 0: 33% *versus* vehicle, 34% *versus* control) and the intermediate striatal sub-region (52% increase *versus* control peptide). These studies suggest that the effect on DA terminals in the striatum is highly selective for A9 nigrostriatal DA projections, which project primarily to the dorsal striatum (Dunnett*, et al.*, 2005). The spatial presentation of the effects of DNSP-11 contrasts previous studies reported by Hebert *et al*., (1996) using GDNF, which showed a uniform increase in DA release at 8 of the 9 recording depths. While the effects of GDNF appear to involve both nigrostriatal (A9) and mesolimbic (A10) DA projections (Dunnett*, et al.*, 2005), DNSP-11 appears to be selective for DA terminals of the dorsal striatum. The pattern of enhanced DA release by DNSP-11 is of interest in the context of PD where DA levels are depleted most severely in the human putamen (Bernheimer*, et al.*, 1973; Hornykiewicz, 1975; Hornykiewicz, 1998; Hornykiewicz, 2002) – the analogous sub-region to the dorsal striatum in rodents (Joel and Weiner, 2000). Additionally, the spatial exclusivity of the enhancing effects of DNSP-11 raises the possibility that previous reports of increased basal DA and DA-metabolite concentrations measured by microdialysis – using a microdialysis probe that samples from nearly the

entire striatum (Bradley*, et al.*, 2010) – are underestimated (if the effect of DNSP-11 on basal DA neurochemistry is also restricted to the dorsal striatum). Also, we would expect the effects on basal neurochemistry (measured at 4 weeks, (Bradley*, et al.*, 2010)) to be greater at the 2-week time point because the current release studies demonstrate that 2-weeks is the peak effect. Although correlation between evoked release and basal neurochemistry is apparent for GDNF (Hebert*, et al.*, 1996), this relationship has not been determined for DNSP-11. A trend in increased release at 4-weeks is not evident in any striatal sub-region after DNSP-11 treatment. However, the increased sensitivity of microdialysis (nanomolar) *versus* the electrochemical release studies (micromolar) may contribute to the discrepancy. Notably, these techniques examine distinct biological processes that should be acknowledged: tonic release (microdialysis – basal levels) and phasic release (electrochemistry – evoked release). Integrating previous studies with the current release data raises important questions about the resulting changes in DA-neuron function after DNSP-11 treatment. It is unclear if changes in basal neurochemistry (DA and metabolites) precede changes in evoked DA release or if these effects are mutually exclusive.

The increase in DA release is a substantial finding that supports functional changes in DA neurons *in vivo*. We believe this finding is relevant because DA levels are markedly reduced in PD (Bernheimer*, et al.*, 1973). Alternatively, enhanced release in the dorsal striatum may be interpreted as merely a consequence of experimental design – using stereotaxic coordinates targeting the SN (see Delivery of DNSPs). However, distribution studies using DNSP-11 and this treatment protocol have demonstrated diffusion of DNSP-11 to the SN as well as the ventral tegmental area (VTA) (Gash *et al.,* unpublished*),* making potential effects of DNSP-11 achievable at both dopaminergic nuclei. Additionally, this infusion method was used previously with GDNF and resulted in a uniform increase in evoked DA at nearly all recording regions of the striatum – supporting modulation DA projections from the VTA and SN (Beckstead*, et al.*, 1979; Bentivoglio and Morelli, 2005; Hebert*, et al.*, 1996). Although distribution studies of GDNF using these methods were not conducted, we predict the larger size and heparin-binding characteristics of GDNF would cause decreased diffusion compared to DNSP-11 (Kelps*, et al.*, 2011; Piltonen*, et al.*, 2009; Salvatore*, et al.*, 2006). Thus DNSP-11 appears to selectively enhance DA release in projections corresponding to the SN despite distribution to adjacent dopaminergic nuclei (the VTA).

The enhanced release phenomena in the striatal sub-region most closely associated with motor function (Bernheimer*, et al.*, 1973; Joel and Weiner, 2000) suggests that DNSP-11 may influence (enhance) locomotor activity. We propose that regional effects of DNSP-11 at dorsal dopaminergic terminals in the striatum reflect a differential effect of DNSP-11 at the cell bodies from which these terminals originate. In particular, these results suggest the SNc is the primary target of DNSP-11 based on specific projections to the dorsal striatum (Beckstead*, et al.*, 1979; Dahlstrom and Fuxe, 1964a; Ungerstedt, 1971), making it a particularly interesting candidate for PD therapy. In contrast, the ventral striatum receives more innervation from the VTA (Beckstead*, et al.*, 1979; Swanson, 1982), where effects of DNSP-11 are not seen at the 2-week time point. These studies cannot directly correlate motor behavior improvements to DNSP-11 treatment based on selectivity of DNSP-11 effects to the motor striatum. Indeed, measures of spontaneous locomotor behavior do not indicate significant changes after DNSP-11 treatment (Fuqua, 2010). Despite the lack of changes in motor activity, we believe DNSP-11 has the potential to modulate motor function. Because non-lesioned F344 rats do not exhibit motor impairments for the age group and parameters used in these studies (Hebert and Gerhardt, 1998), behavioral endpoints would be more appropriately examined in a model with a behavioral deficit. For example, in rats with 6-hydroxydopamine lesions, DNSP-11 has shown improved neurochemical function with corresponding behavioral improvement (Bradley*, et al.*, 2010). Thus, the behavioral significance of DNSP-11 should be further examined in systems with a neurochemical deficiency of the nigrostriatal pathway and a corresponding behavioral deficit.

DNSP-5/DNSP-11 Combination Treatment Enhances Tissue Neurochemical Content but not Extracellular Levels

Because the post-translational processing model of GDNF predicts DNSP-11 and DNSP-5 are generated simultaneously, neurochemical studies were conducted to determine if treatment with a 2-peptide mixture (DNSP-5 + DNSP-11) demonstrated biological effects *in vivo.* Extracellular basal levels of DA, DOPAC and HVA were not changed by DNSP-5/DNSP-11 treatment ($p > 0.05$, all). Interestingly, the peptide mixture increased tissue levels of DA, DOPAC and HVA (69%, 207%, and 52%, respectively *versus* vehicle) in the SN and decreased (23%) tissue levels of HVA in the dorsal striatum. These experiments were designed to reproduce previous studies using a 3-peptide mixture (10 µg each: DNSP-5 + DNSP-11 + DNSP-17), which contained an

additional 17-amino-acid peptide (DNSP-17) derived from the pro-region of GDNF (Fuqua, 2010; Kelps*, et al.*, 2011). The 3-peptide mixture increased basal extracellular levels of DA, DOPAC and HVA and also increased TH-positive fiber density in the SN. The basal neurochemical and histological findings were not accompanied with changes in tissue neurochemical content (Fuqua, 2010). We would predict an increase in DA-neuron fiber density in the SN to underlie the increases in DA and metabolites in the DNSP-5/DNSP-11 studies based on findings with the 3-peptide mixture. The discrepancy in results from the 2- and 3-peptide mixtures suggests diverse signaling and likely distinct cellular mechanisms among the three related peptides. Based on association with metabolic proteins (Bradley*, et al.*, 2010), the cellular mechanism(s) linked to DNSP-11 are suspected to involve mitochondrial function. However, different mechanisms are suggested for the other DNSPs (Kelps*, et al.*, 2011). Distinct cellular mechanisms are supported by the lack of intermolecular interaction among the three peptides *in vitro* and variable protection from mitochondrial toxins (staurosporine and 3-nitropropionate) (Kelps*, et al.*, 2011) among the three peptides. Conceivably, the actions of the DNSPs (in combination or alone) may also be modulated by GDNF, a possibility requiring further investigation.

As described above, DNSP-5 and DNSP-11 are believed to act independently (Kelps*, et al.*, 2011). The dose used for co-injection of DNSP-5 and DNSP-11 should be reconsidered because an equal mass solution (DNSP-5 + DNSP-11) contains a disproportionate number of each peptide. It is possible that DNSP-5 acts by an unidentified negative feedback mechanism, opposing the effects of DNSP-11. The effects of the peptide mixture may be more appropriately investigated using an equal molar solution. However, neurochemical effects on extracellular neurochemistry (without changes in tissue content) are apparent using the same dose in the 3-peptide mixture (Fuqua, 2010), suggesting an important role of the 17-amino-acid peptide. The specific cellular actions of the peptides (individual and combinations) are largely speculative at this time.

Neurochemical Effects of DNSPs Support Unique Function of Pro-peptides

To our knowledge DNSP-11 is the first neurotrophic factor-derived pro-peptide to be investigated for neurotrophic actions to this extent. Trophic-like effects of pro-peptides are predicted because pro-peptides have been implicated in cell survival pathways (Ibanez, 2002; Lee*, et al.*, 2001). Interestingly, uncleaved neurotrophins (e.g.

pro-BDNF) have been associated with the induction of apoptotic pathways. By contrast, the cleaved neurotrophin (BDNF) induces cell survival- and differentiation-pathways (Teng*, et al.*, 2005). The function of pro-BDNF may also be modulated by the proteolytic processing of a different neurotrophin – NGF (Dicou, 2006; Dicou*, et al.*, 1997). Thus processing of particular neurotrophins may not only determine the cellular pathway(s) activated but may also regulate activity of other (different) neurotrophins. These findings further substantiate the importance of post-translational processing and suggest possible interactions between neurotrophins and pro-neurotrophins.

The biological activity of the DNSPs, pro-peptides, is particularly interesting in the context of differential splicing of GDNF. There are two known isoforms of GDNF with one isoform containing a truncated (26-amino-acid) pro-region (Grimm*, et al.*, 1998). Un-cleaved (pro-GDNF) and mature forms of both isoforms have demonstrated distinct cellular localization. In addition, secretion of the individual isoforms demonstrates differential modulation by neuronal activity (Lonka-Nevalaita*, et al.*, 2010). In the context of the presented studies, which show effects on depolarization-induced release after DNSP-11 treatment, the action of pro-peptides on neuronal activity merits further investigation to determine the relationship, if any, between neuronal activity and pro-GDNF processing and secretion. In summary, post-translational processing of pro-proteins and the resulting generation of multiple bioactive peptides may activate diverse signaling cascades (Bernay*, et al.*, 2009; Haskins*, et al.*, 2004) that may be interrelated. Applied to the present findings, we expect further investigation of signaling pathways may reveal activation of converging pathways associated with cell survival and differentiation (Ibanez, 2002; Lee*, et al.*, 2001) that are initiated distinctly by the DNSPs and GDNF (Bradley*, et al.*, 2010; Kelps*, et al.*, 2011).

DNSP-11 is the first peptide from the prodomain of the GDNF family to demonstrate *in vivo* trophic-like actions with potential therapeutic value in treating neurodegenerative disease (Bradley*, et al.*, 2010; Immonen*, et al.*, 2008). The comparably long–lasting effect (weeks) of a single administration of DNSP-11 is promising given plasma levels of current PD therapies like L-DOPA peak within one hour (Adamiak*, et al.*, 2010; Harder and Baas, 1998) and are largely unsuccessful in treating underlying disease pathology/progression (Kempster*, et al.*, 2007; Marsden and Parkes, 1977). Although the symptomatic relief provided with PD drug therapies may be sustained for weeks after drug withdrawal (Fahn*, et al.*, 2004), the majority of patients undergoing chronic L-DOPA therapy experience undesirable side effects like

dyskinesias along with reduced efficacy within a few years after starting treatment (Ahlskog and Muenter, 2001; Rajput*, et al.*, 2002). Because dyskinetic side effects have been attenuated with trophic factor treatment in animal models (Costa*, et al.*, 2001; Iravani*, et al.*, 2001), the molecular and cellular changes that are suspected to underlie problems with chronic medical treatment (Nutt, 2003; Obeso*, et al.*, 2000) may be appropriate targets for DNSP-11 treatment. Thus, in addition to the trophic-like, potentially disease-progression modifying effects of DNSP-11, DNSP-11 may be a valuable adjunct therapy to extend the effective therapeutic window for existing medical treatments.

Conclusions

Although pro-peptides were previously thought to function only to assist in the processing and secretion of the mature neurotrophin (Rattenholl*, et al.*, 2001), these studies support specific trophic-like effects on the nigrostriatal pathway. In conclusion, the use of DNSP-11 and/or related peptides could advance the treatment of neurodegenerative disease markedly by preventing neuronal loss and/or restoring function of damaged neurons (Grondin and Gash, 1998; Lapchak*, et al.*, 1997a; Peterson and Nutt, 2008). Because of their relative size and less complex structure, DNSP-5, DNSP-11 and other small peptides with trophic-like actions may provide specific advantages over larger molecules including: ease of synthesis and long-term storage, improved biodistribution/lack of heparin binding (Kelps*, et al.*, 2011) and potential for enhanced bioavailabilty and/or modification to allow alternative (less invasive) delivery routes (Bespalov and Saarma, 2007). The possibility for alternative administration routes provides substantial advantages over treatment regimens required for GDNF and other neurotrophic factors, which involve intracranial delivery by pump or gene therapy approaches (Bjorklund*, et al.*, 2000; Gill*, et al.*, 2003; Grondin*, et al.*, 2002; Patel and Gill, 2007). These studies demonstrate the neurotrophic-like functional properties of DNSP-11 and suggest that the DNSPs may be one of many overlooked groups of bioactive pro-peptides that merit continual examination and evaluation for potential therapeutics. With favorable physical and physiological properties, further studies are warranted to evaluate DNSPs as therapeutic compounds for neurodegenerative diseases like PD.

Chapter Three: Figures

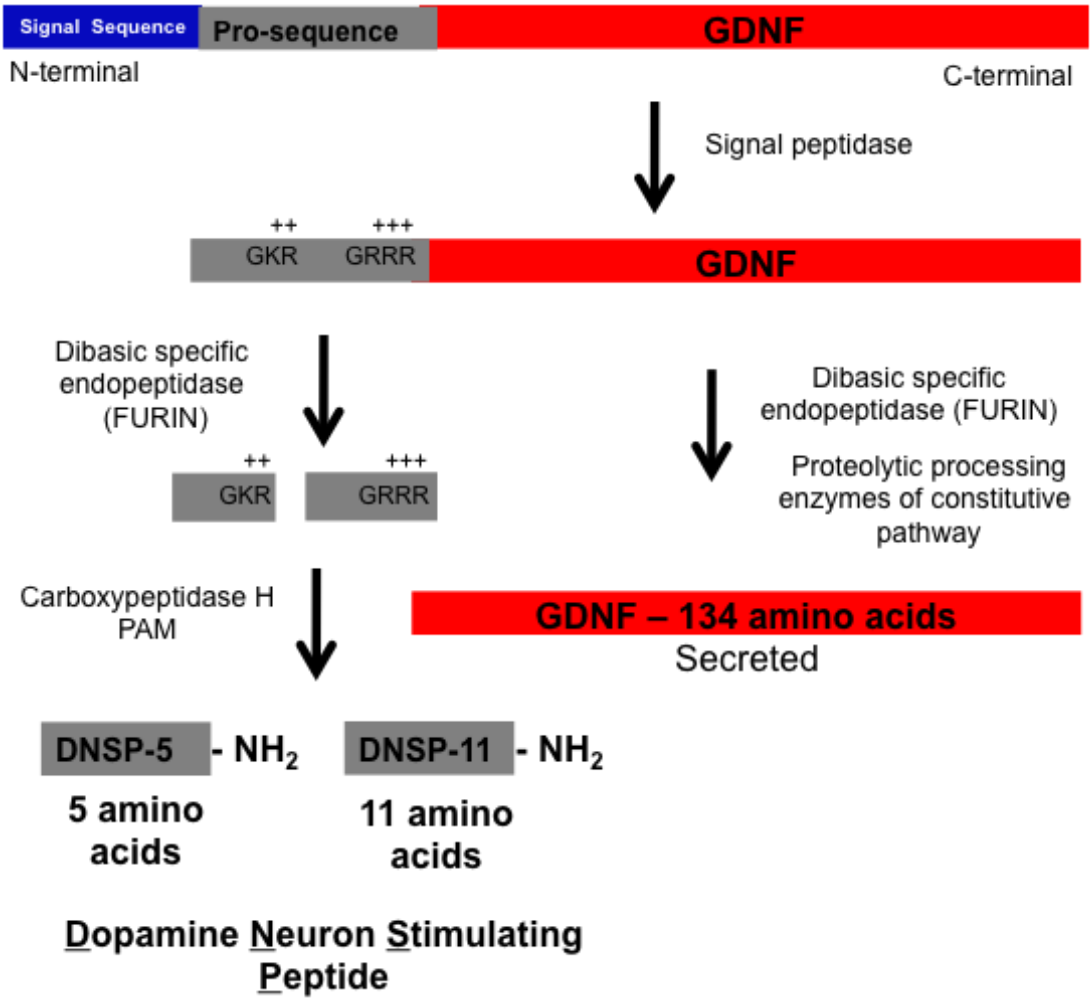

Figure 3.1 Post-translational Processing Model of GDNF
The pre-pro-GDNF molecule is shown (not drawn to scale) where the peptides of interest, DNSP-5 and DNSP-11, originate from pro-region (gray). After cleavage of the signal sequence (blue), a series of enzymatic reactions on the pro-GDNF sequence (gray) are predicted to occur *in vivo*. The actions of specific endopeptidases cleave the pro-protein region at C-terminal, paired-basic amino-acid (++) residues. The resulting pro-proteins undergo further processing at the C-terminal regions. The actions of carboxypeptidase H and peptidylglycine amidating monooxygenase (PAM) yield DNSP-5 and DNSP-11 – both amidated at the C-terminus. Modified from Bradley *et. al.,* 2010.

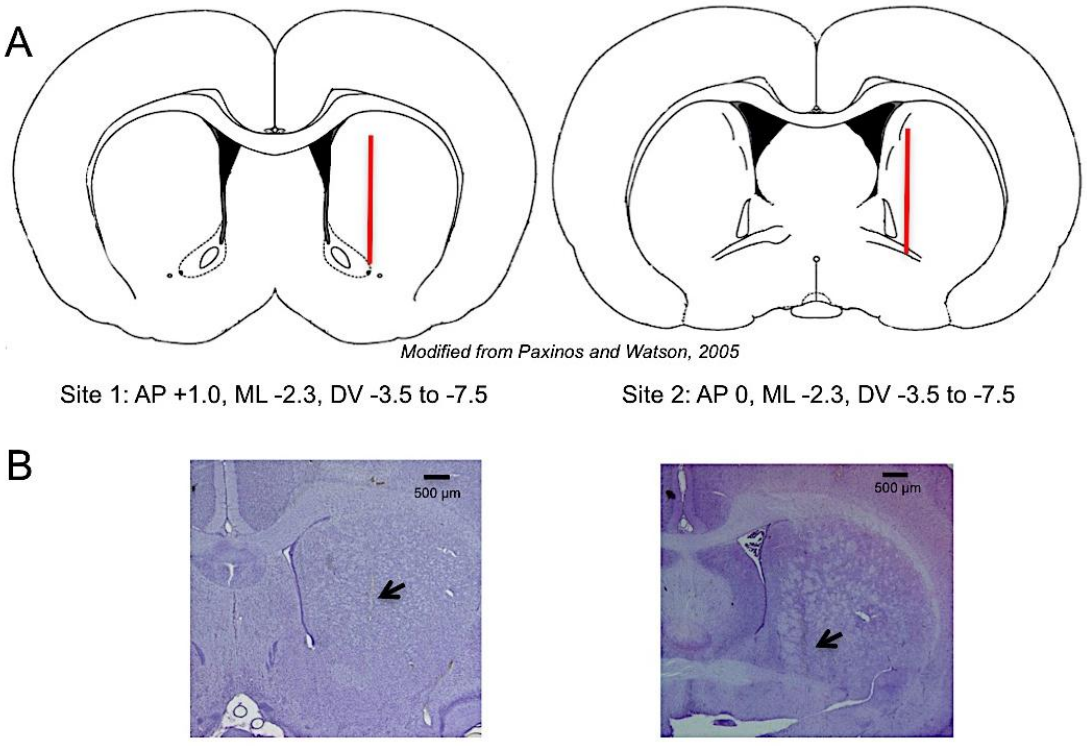

Figure 3.2 Microelectrode Placement in the Rat Striatum
The figures are representative of the observed recording sites corresponding to stereotaxic coordinates (A). Two striatal recording sites were used for electrochemical recordings (from *bregma* (mm)): Site 1 (rostral site) and Site 2 (caudal site). One DA signal was elicited at 500 µm increments along the dorsal-ventral axis of the striatum for each recording site in the treated (right) hemisphere. Histological sections prepared after electrochemcial recordings are shown (B). The most clearly identified portion of the electrode tract is indicated (arrow). Because of minimal tissue disruption using a small recording electrode (~30 µm o.d.), the most ventral placement of the electrode could not be visualized in all brain slices.

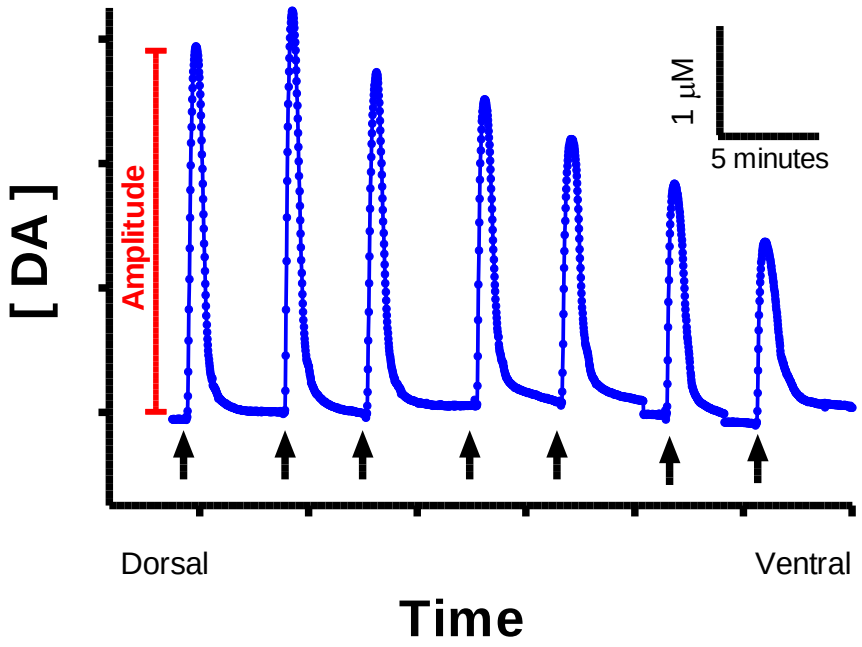

Figure 3.3 Evoked Release of Dopamine in the Rat Striatum
A potassium chloride solution is applied locally (120 mM, arrow) to elicit one DA signal every 500 µm along the dorsal-ventral axis of the striatum. The amplitude is calculated as the change from baseline for each signal. Dopamine release may be very heterogeneous in the striatum because of differences in DA terminal density. Inset: axes units.

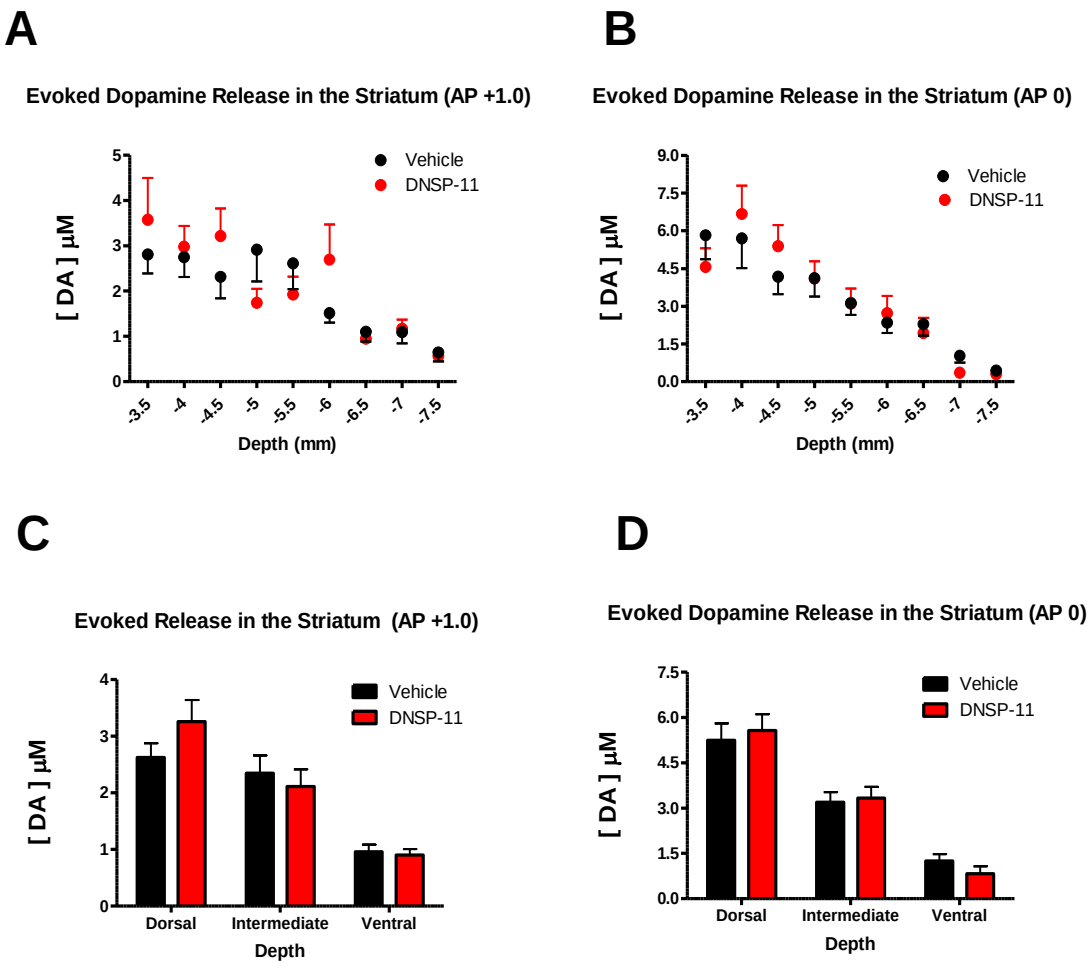

Figure 3.4 Striatal Depth Profiles of Evoked Dopamine Release - 1 Week after Treatment

Using carbon-fiber microelectrodes with high-speed chronoamperometry, measurements of DA release from local application of potassium were taken. One DA signal was elicited at 500 µm increments in the rat striatum. The average DA-signal amplitude from 2 striatal recording sites is shown for DNSP-11 and vehicle treated animals (A)(B). The DA-signal amplitudes from 3 recording depths were combined to determine the mean release amplitude for striatal sub-regions (C)(D) (dorsal, intermediate and ventral). There were no statistically significant differences in signal amplitude between treatment groups for any of the recording depths or striatal sub-regions. Data are shown as mean ± SEM. All data were analyzed using a two-way ANOVA with Bonferroni's *post hoc* tests.

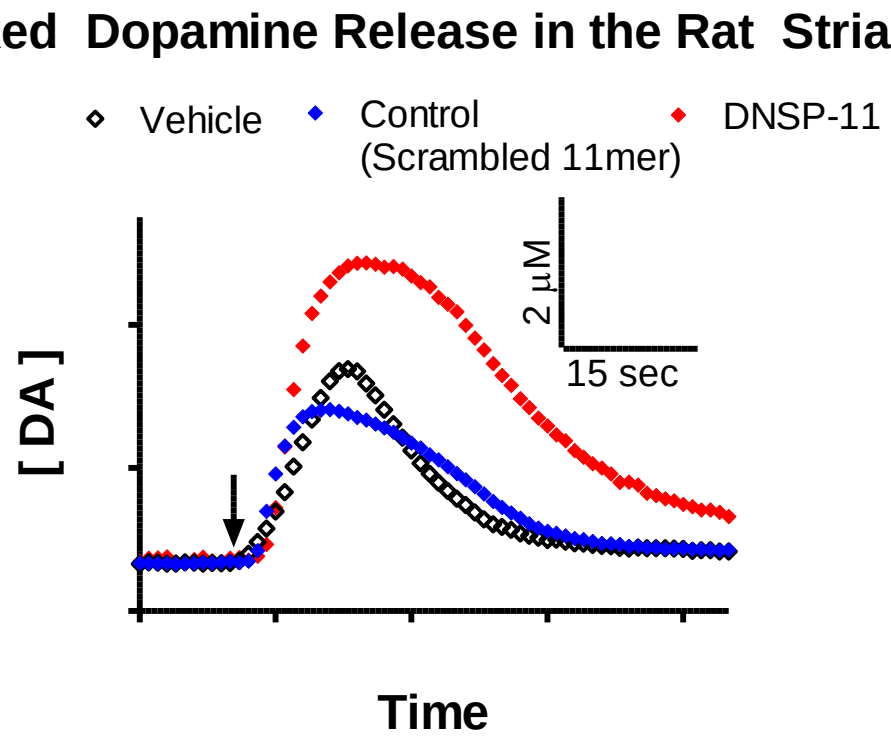

Figure 3.5 Evoked Dopamine Release is Increased – 2 Weeks after DNSP-11 Treatment Representative traces of DA-release amplitude in the dorsal striatum evoked by local application of potassium (arrow) are shown. Vehicle, Control and DNSP-11 treatment groups are shown 2 weeks after a single intranigral treatment (inset: axes units). DNSP-11 treatment (red) increased the amplitude of DA release compared to vehicle (open) and control (blue) treated animals.

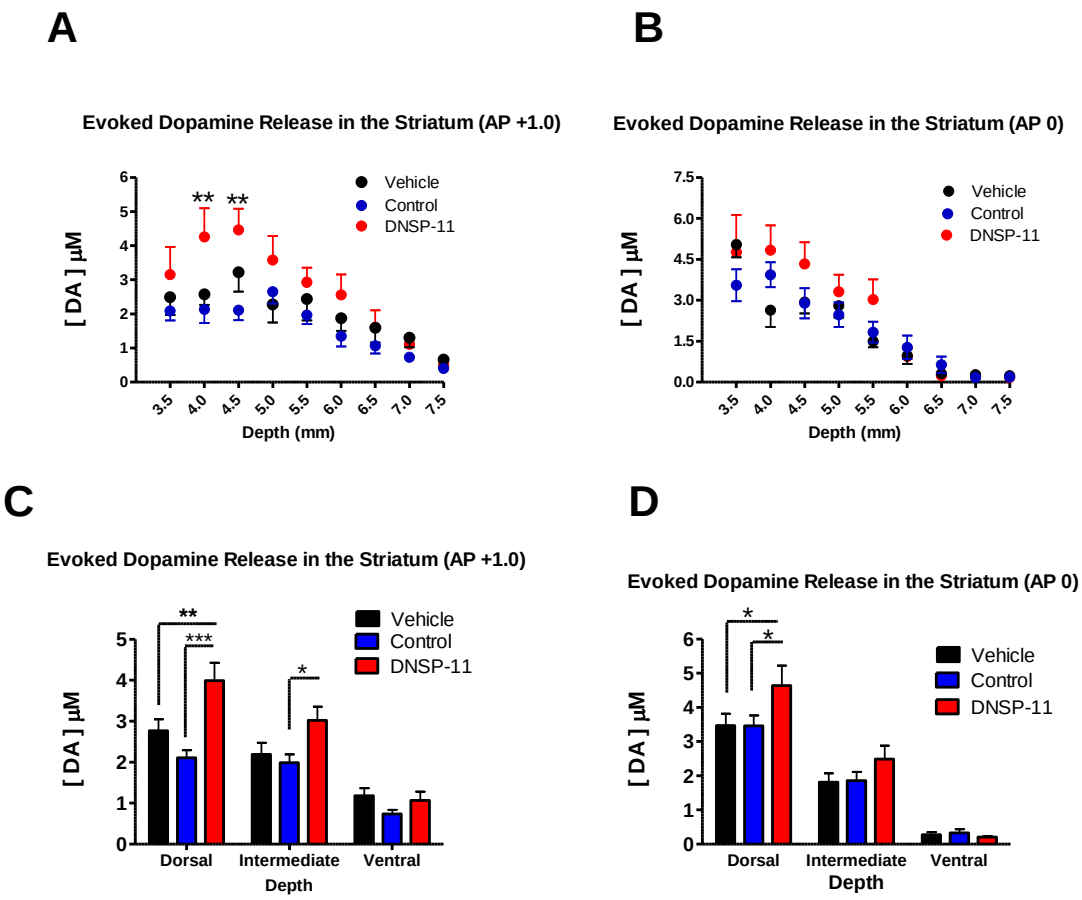

Figure 3.6 Striatal Depth Profiles of Evoked Dopamine Release - 2 Weeks after Treatment

Using carbon-fiber microelectrodes with high-speed chronoamperometry, measurements of DA release from local application of potassium were taken. One DA signal was elicited at 500 µm increments in the rat striatum. The average DA-signal amplitude from 2 striatal recording sites is shown for DNSP-11, control and vehicle treated animals **(**A)(B). A significant effect of treatment was observed in the rostral (AP+1.0) recording site (A). *Post hoc* analysis indicates a significant increase in DA release in DNSP-11 treated groups *versus* the control peptide at two individual recording depths in the striatum (A) [DV: 4.0 mm, $^{**}p < 0.01$, DV: 4.5 mm, $^{**}p < 0.01$, $n = 8$]. The DA signal amplitudes from 3 recording depths were combined to determine the mean release amplitude for striatal sub-regions (C)(D) (dorsal, intermediate and ventral; *n* = number of signals). There was a significant effect of treatment in both striatal recording sites. In the rostral recording site (C) *post hoc* analysis confirms a significant increase in amplitude in DNSP-11 treated groups *versus* vehicle ($^{**}p < 0.01$, $n = 23$) and control peptide ($^{***}p <$

0.001, *n* = 23-24) in the dorsal striatum. In the intermediate striatum, there was also a significant increase (**p* < 0.05, *n* = 23-24) in amplitude in DNSP-11 treated groups *versus* control peptide. In the caudal recording site (D) *post hoc* analysis indicates a significant increase in amplitude in DNSP-11 treated groups (*n* = 24) in the dorsal striatum *versus* vehicle (**p* < 0.05, *n* = 23) and control (**p* < 0.05, *n* = 24). Data are shown as mean ± SEM. All data were analyzed using a two-way ANOVA with Bonferroni's *post hoc* tests.

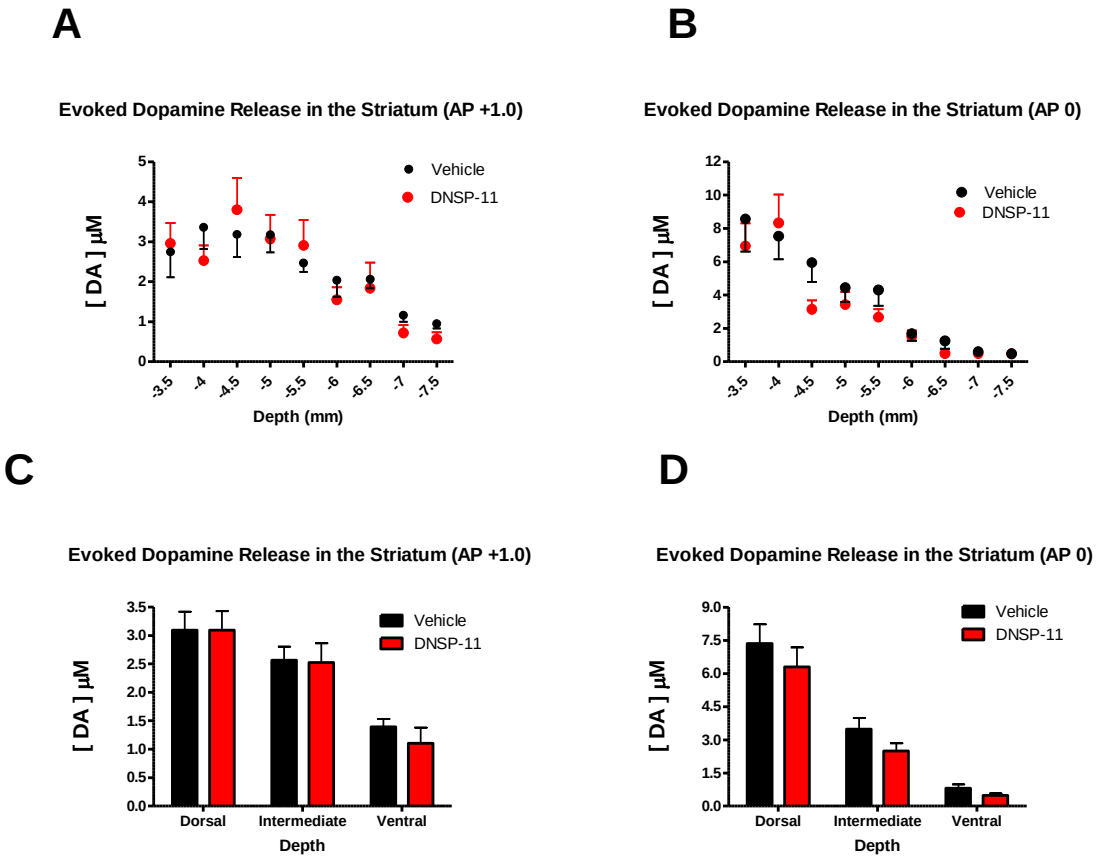

Figure 3.7 Striatal Depth Profiles of Evoked Dopamine Release - 4 Weeks after Treatment

Using carbon-fiber microelectrodes with high-speed chronoamperometry, measurements of DA release from local application of potassium were taken. One DA signal was elicited at 500 µm increments in the rat striatum. The average DA-signal amplitude from 2 striatal recording sites is shown for DNSP-11, and vehicle treated animals (A)(B). The DA signal amplitudes from 3 recording depths were combined to determine the mean release amplitude for striatal sub-regions (C)(D) (dorsal, intermediate and ventral). There were no statistically significant differences in signal amplitude between treatment groups for any of the recording depths or striatal sub-regions. Data are shown as mean ± SEM. All data were analyzed using a two-way ANOVA with Bonferroni's *post hoc* tests.

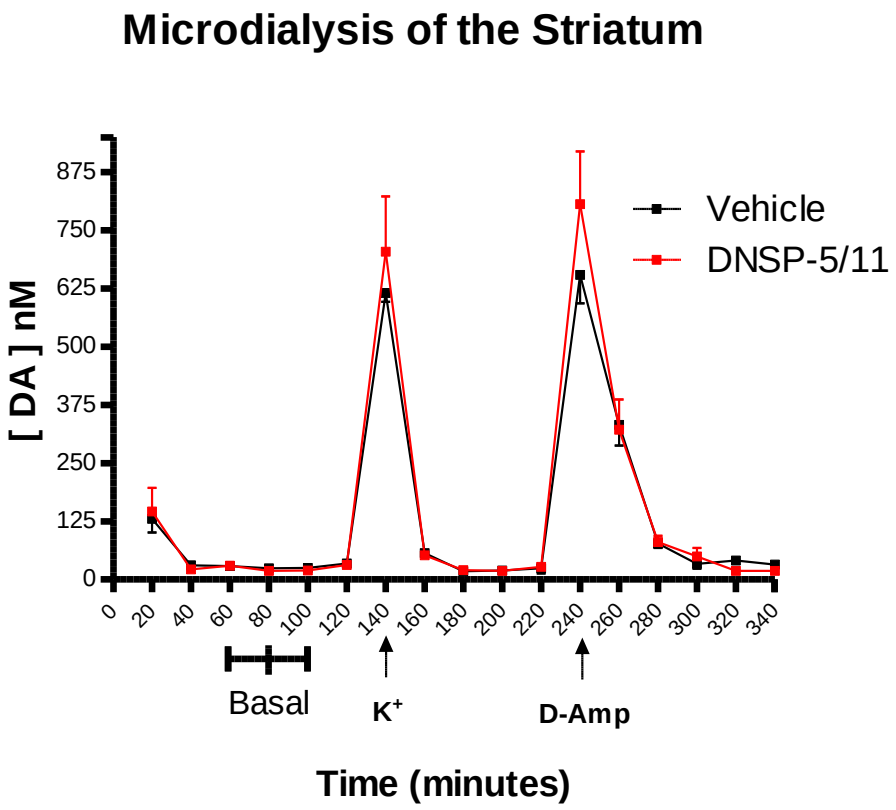

Figure 3.8 *In Vivo* Microdialysis after DNSP-5/-11 Treatment
Basal extracellular levels of DA (shown) and DA metabolites were determined by striatal microdialysis following intranigral DNSP-5/-11 or vehicle treatment. Microdialysis samples were collected at 20-minute intervals. The average of three samples was calculated to determine basal levels of DA and DA metabolites. High-potassium (K^+) and d-amphetamine (D-Amp) aCSF solutions were used to stimulate DA release. Data are shown as mean ± SEM.

Table 3.1 DNSP-11 and DNSP-5 Sequences

Species	DNSP-11	DNSP-5
Human	Pro-Pro-Glu-Ala-Pro-Ala-Glu-Asp-Arg-Ser-Leu	Phe-Pro-Leu-Pro-Ala
Rat	Leu-Leu-Glu-Ala-Pro-Ala-Glu-Asp-His-Ser-Leu	Phe-Pro-Leu-Pro-Ala
Mouse	Leu-Leu-Glu-Ala-Pro-Ala-Glu-Asp-His-Ser-Leu	Phe-Pro-Leu-Pro-Ala

DNSP-11 and DNSP-5 sequences are highly conserved among related species. Both peptides are generated through the predicted post-translational processing of the pro-region of GDNF.

Table 3.2 Tissue Neurochemical Content after Treatment with DNSP-11

Brain Region and Treatment	DA	DOPAC	HVA	DOPAC:DA x 10^3	HVA:DA x 10^3	(DOPAC + HVA) : DA x 10^3
Dorsal Striatum						
Vehicle	12910 ± 1422	3186 ± 369	756 ± 96	272.5 ± 52.9	63.75 ± 12	336.5 ± 64
DNSP-11	10400 ± 1753	3254 ± 503	589 ± 77	374.5 ± 68.3	62.38 ± 6.9	437.0 ± 75
Ventral Striatum						
Vehicle	8363 ± 1482	3623 ± 672	715 ± 118	470.0 ± 55.3	97.13 ± 12.4	566.9 ± 67.3
DNSP-11	8690 ± 1433	3149 ± 343	676 ± 50	441.4 ± 85.7	93.00 ± 16.3	534.3 ± 101
Substantia nigra						
Vehicle	562 ± 92	150 ± 42	111 ± 28	156.4 ± 23	148.5 ± 23	343.7 ± 72
DNSP-11	995 ± 316	269 ± 108	131 ± 50	186.2 ± 46.7	139.3 ± 35	370.1 ± 92

Data shown as mean ± SEM (ng/g wet weight of tissue). Analyzed by a two-tailed unpaired *t* test comparing treatment groups.

Table 3.3 Extracellular Levels of Dopamine and Metabolites after Treatment with Peptide Mixture (DNSP-5 and DNSP-11)

Treatment	DA	potassium-evoked DA	d-amphetamine-evoked DA	DOPAC	HVA
Vehicle	26 ± 1.1	616 ± 19	654 ± 61	4335 ± 517	3701 ± 632
DNSP-5/-11	23 ± 4.8	704 ± 118	806 ± 114	3312 ± 672	2519 ± 397

Data shown as mean ± SEM (nM). Analyzed by a two-tailed unpaired *t* test comparing treatment groups.

Table 3.4 Tissue Neurochemical Content after Treatment with Peptide Mixture (DNSP-5 and DNSP-11)

Brain Region	Treatment	DA	DOPAC	HVA	DOPAC:DA x10^3	HVA:DA x10^3	(DOPAC + HVA): DA x10^3
Dorsal Striatum	Vehicle	12774 ± 1177	4213 ± 81	1088 ± 73	338.3 ± 32	87.50 ± 10	425.8 ± 41
	DNSP-5/-11	12322 ± 743	4581 ± 801	838 ± 57* $t(6) = 2.686$, $^*p = 0.0362$	374.3 ± 62.1	68.8 ± 5.3	443 ± 65
Ventral Striatum	Vehicle	10953 ± 1077	3446 ± 285	987 ± 59	319.8 ± 32.3	92.5 ± 11	413 ± 42
	DNSP-5/-11	8602 ± 1547	3538 ± 1063	1121 ± 264	448.5 ± 123.1	140.0 ± 29.0	588.5 ± 152
Substantia nigra	Vehicle	284 ± 92	58 ± 11	93 ± 22	242 ± 44	534 ± 308	777 ± 350
	DNSP-5/-11	925 ± 214* $t(6) = 2.754$, $^*p = 0.0331$	178 ± 19** $t(6) = 5.476$, $^{**}p = 0.0015$	192 ± 26* $t(6) = 2.909$, $^*p = 0.0270$	208 ± 26	220 ± 19	428 ± 44

Data shown as mean ± SEM (ng/g wet weight of tissue). Analyzed by a two-tailed unpaired *t* test comparing treatment groups.

Chapter Four: Dynamic Changes in Nigrostriatal Dopamine-Neuron Function in *Gdnf*$^{+/-}$ Mice – A Novel Model of Nigrostriatal Dysfunction

Introduction

The decline in motor function associated with aging has been demonstrated in animal models of aging (Hebert and Gerhardt, 1998; Willig*, et al.*, 1987; Yurek*, et al.*, 1998; Zhang*, et al.*, 2000) and parallels a similar decline in human aging (Bennett*, et al.*, 1996; Kluger*, et al.*, 1997; Richards*, et al.*, 1993). Dopamine-neuron dysfunction has been linked to age-associated motor impairment in humans (Volkow*, et al.*, 1998b) and animals (Gerhardt*, et al.*, 2002; Hebert and Gerhardt, 1998; Yurek*, et al.*, 1998). The loss of functional DA neurons in the SNc and consequent loss of striatal DA are hallmarks of PD (Marsden, 1990), which presents with cardinal motor symptoms: bradykinesia, rigidity, postural instability and tremor (Hornykiewicz and Kish, 1987; Parkinson, 2002). The presence of some of these deficits is observed in many aged individuals and has been termed "age-associated parkinsonism" (Bennett*, et al.*, 1996), which likely involves changes in the functional properties of DA neurons rather than marked neuronal loss as demonstrated in aged rats and non-human primates (Grondin*, et al.*, 2003; Hebert and Gerhardt, 1999; Hebert*, et al.*, 1999; Yurek*, et al.*, 1998).

Glial cell line-derived neurotrophic factor (GDNF) is a distant member of the transforming growth factor-β superfamily (Lin*, et al.*, 1993). It is hypothesized that age-related decreases in neurotrophic factor levels contribute to DA-neuron degeneration and/or dysfunction (Yurek and Fletcher-Turner, 2001). *In vivo* application of exogenous GDNF to the SN has been reported to enhance DA-neuron function in young (Hebert*, et al.*, 1996), aged (Grondin*, et al.*, 2003; Hebert and Gerhardt, 1997) and lesioned (Hoffer*, et al.*, 1994; Tomac*, et al.*, 1995) animals. In addition, GDNF protects and restores DA-neuron function related to neurotoxic doses of methamphetamine in rats (Cass*, et al.*, 2000; Cass*, et al.*, 2006) and restores function in rhesus monkeys with MPTP-induced parkinsonism (Gash*, et al.*, 1996; Grondin*, et al.*, 2002). Treatment with GDNF has also been shown to enhance DA-neuron function indicated by increased: 1) evoked DA release 2) locomotor behavior 3) DA content in nigral tissue (Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996) and 4) high-affinity DA uptake (Lin*, et al.*, 1993). However, the exact role(s) of GDNF in normal aging, specifically the maintenance of DA-neuron function, is not known.

At birth, midbrain DA systems in mice homozygous for the GDNF null mutation (*Gdnf*$^{-/-}$) appear normal (Moore*, et al.*, 1996), however, function of these neurons after major apoptotic waves (occurring 2 and 14 days after birth) (Mahalik*, et al.*, 1994) cannot be assessed because these mice die at birth due to kidney agenesis (Moore*, et al.*, 1996). Therefore, postnatal *in vivo* studies have used mice with a partial deletion of the *Gdnf* gene (*Gdnf*$^{+/-}$) (Pichel*, et al.*, 1996), which exhibit a partial and stable reduction of GDNF protein in brain tissues (Boger*, et al.*, 2006). GDNF expression in the striatum appears to be critical for the survival and differentiation of midbrain DA neurons and innervation of striatal targets during early development (Stromberg*, et al.*, 1993). Indeed, postnatal development of these DA neurons is compromised in the absence of GDNF (Granholm*, et al.*, 2000). Behavioral and immunohistochemical characterization in multiple age groups (4-, 8-, 12-, 16- and 20-month-old) provides evidence for a unique aging phenotype in the *Gdnf*$^{+/-}$ mice. A partial GDNF depletion leads to an earlier loss of TH-positive neurons in the SNc and diminished spontaneous locomotor activity at 12 months of age (Boger*, et al.*, 2006). The diminished locomotor activity and decline in TH-positive neurons also demonstrate an accelerated decline with age (between 8 and 12 months) in the *Gdnf*$^{+/-}$ mice. By contrast, both measures in WT mice of the same age groups are unchanged (Boger*, et al.*, 2006) and young *Gdnf*$^{+/-}$ mice (6 to 9-month-old) do not exhibit changes in spontaneous or methamphetamine-induced locomotor behavior (Boger*, et al.*, 2006; Boger*, et al.*, 2007; Gerlai*, et al.*, 2001). *In vitro* studies using synaptosomal preparations from *Gdnf*$^{+/-}$ mice indicate increased DAT activity that has been suggested to predispose DA neurons to methamphetamine-induced toxicity (Boger*, et al.*, 2007). Consistent with the age-related changes in DA systems noted above, DAT activity in the nigrostriatal pathway is altered in the normal aging process in animal models (Friedemann, 1992; Friedemann and Gerhardt, 1992; Hebert and Gerhardt, 1999; Hebert*, et al.*, 1999) and may be correlated with age-related motor deficits. Dynamic changes in DA uptake have been characterized by *in vivo* chronoamperometry using carbon fiber microelectrodes and parameters associated with DAT activity (Cass*, et al.*, 1993; Gerhardt*, et al.*, 1999; Gerhardt*, et al.*, 1986). The high spatial (microns) and temporal (seconds) resolution (Gerhardt and Hoffman, 2001) of this electrochemical technique allows for the rapid and sensitive quantification of DA uptake in discrete DA-terminal sub-regions.

Based on *in vitro* studies that indicate DA uptake in *Gdnf*$^{+/-}$ mice was increased compared to WT mice (Boger*, et al.*, 2007), the present study investigated this age-

associated abnormality by using *in vivo* electrochemistry to examine the effects of a partial and chronic genetic reduction of GDNF on the function of DA terminals in the striatum of middle-aged (12-month-old) *Gdnf*$^{+/-}$ mice. Additionally, because GDNF supplementation has been shown to enhance DA release in the rat and non-human primate (Grondin*, et al.*, 2003; Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996), DA release in middle-aged *Gdnf*$^{+/-}$ mice was investigated. The following questions were addressed: (1) Do *Gdnf*$^{+/-}$ mice differ in behavioral response to DAT modulation via DAT inhibition? (2) Do *Gdnf*$^{+/-}$ mice exhibit altered DA-clearance and -release properties *in vivo* and what is the spatial pattern of this effect within striatal sub-regions? (3) Do *Gdnf*$^{+/-}$ mice have altered striatal DA-receptor expression? (4) Is whole tissue neurochemical content altered in *Gdnf*$^{+/-}$ mice?

Collectively, these studies focus on the functional properties of DA neurons in 12-month-old *Gdnf*$^{+/-}$ mice, which demonstrate accelerated age-related motor deficits and loss of TH-positive neurons (Boger*, et al.*, 2006), with the hypothesis that GDNF depletion causes *in vivo* alterations in DA-neuron function that contribute to locomotor function.

Materials and Methods

Electrochemical Recordings

For electrochemical recordings, *Gdnf*$^{+/-}$ (n = 5) and WT littermates (n = 7) were prepared as described (see In Vivo Electrochemical Recording and Microdialysis – Non-Survival Surgical Procedures).

The microelectrodes used for electrochemical recordings were prepared (see In vivo Electrochemistry) and chronoamperometric measures were carried out as described previously (See Chronoamperometry). Calibration of microelectrodes confirmed linear responses to serial additions of DA (2 – 6 µM, effective beaker concentrations) with correlation coefficients (R^2) ≥ 0.99. Selectivity of the electrodes for DA (*versus* ascorbate) averaged 908:1 ± 166 (n = 13). The LOD (nM) for DA was 11 ± 3 (n = 13) with a signal to noise ratio ≥ 3. Calibration parameters are expressed as mean ± SEM, n = number of electrodes.

Two striatal recording sites per hemisphere were used to determine DA-uptake and -release characteristics. Stereotaxic coordinates from *bregma* were (mm): Site 1 (rostral-medial): anterior-posterior +1.0, medial-lateral +/- 1.2, dorsal-ventral -3.0 to -4.6; Site 2 (caudal-lateral): anterior-posterior +0.1, medial-lateral +/- 2.2, dorsal-ventral -2.5

to -4.1 (incisor bar positioned with *lambda* level to *bregma*; Figure 4.1) (Franklin and Paxinos, 2001). The electrode recording assembly was stereotaxically placed into the targeted striatal recording sites. The recording assembly was lowered at 400 µM increments through the dorsal-ventral orientation of the striatum. Dopamine uptake characteristics were determined by local application (see Local Application of Solutions In Vivo) of DA solution (100 µM DA, 200 µM ascorbate, in 0.9% NaCl) at each depth in the left hemisphere. Dopamine release was investigated similarly, by local application of potassium solution (120 mM KCl, 29 mM NaCl, 2.5 mM $CaCl_2 \cdot 2H_2O$) (Gerhardt*, et al.*, 1986) in the right hemisphere.

After initial placement of the microelectrode/micropipette assembly in the brain, cortical baseline recordings were conducted for a minimum of 1 hour. The recording assembly was then lowered to the targeted recording area in the striatum for studies of DA-uptake and -release. Signals analyzed for DA release were volume matched (75-150 nL) for the amount of potassium solution applied. For DA uptake studies, the solution volume was adjusted and recorded in order to obtain DA signals of equivalent amplitudes, which were analyzed for clearance kinetics (Cass and Gerhardt, 1995; Cass*, et al.*, 1993). In both the release and uptake studies, a single DA signal was elicited at several depths along the dorsal-ventral orientation of the striatum. When the signal had returned to a stable baseline, the recording assembly was lowered 400 µm to the next recording depth. Baseline recordings were collected for a minimum of 5 minutes before local application of solution at the next recording depth. All DA signals used for analysis exhibited mean reduction:oxidation ratios consistent with the established profile for DA (mean ± SEM; DA uptake studies: 0.78 ± 0.01 (n = 107), DA release studies: 0.779 ± 0.01 (n = 98); n = number of signals) (Gerhardt and Hoffman, 2001; Gerhardt*, et al.*, 1986).

Analysis of Electrochemical Recordings

Several signal parameters obtained from individual DA signals were analyzed (Figure 4.2). High–speed chronoamperometric data were analyzed using custom MATLAB®-based software (The MathWorks™, Inc.). Signal amplitude was calculated as the current change (Δ) from baseline after local application of solutions. The change in current can be divided by the calibration slope to determine the effective change in DA in concentration units: µM (release studies) or nM (uptake studies). Signal amplitude was calculated for both uptake and release studies and was the primary parameter of

interest in the release studies. In the DA uptake studies, the first order exponential decay constant, k_{-1} (sec^{-1}), was calculated from the decay portion of the signal fitted to the slope of the linear regression of the natural log transformation of the data (Sabeti*, et al.*, 2002). The first order decay constant, k_{-1} (sec^{-1}), is a quantitative measure that can be multiplied by the signal amplitude (nM) to yield the uptake rate (nM/sec) (Stephens*, et al.*, 2009). For our purposes, we analyzed only the k_{-1} because signal amplitudes were matched. A single value for k_{-1} was obtained using one signal from each recording depth using signal amplitudes that were matched (Cass*, et al.*, 1992; Cass*, et al.*, 1993). The volume of solution applied to obtain signals with amplitudes ranging from 500-1000 nM was recorded and used to calculate the total amount (pmol) of DA applied. Adjusting the volume of DA applied enables modulation of signal amplitude such that physiologically appropriate concentrations are analyzed and the inherent Michaelis-Menten properties of the DAT are not altered by varied extracellular concentrations of substrate (Hebert and Gerhardt, 1999). In addition, the DA signal amplitude (nM) per amount of DA applied (pmol) (Figure 4.2) is a measure that reflects surface expression of the DAT (Cass and Gerhardt, 1995; Hebert and Gerhardt, 1999). Because prior work demonstrated heterogeneous properties of DA uptake and DAT density, which varies most notably along the dorsal-ventral orientation of the striatum (Hebert and Gerhardt, 1999; Hebert*, et al.*, 1999), uptake data (k_{-1} and amplitude per picomole DA applied) from the striatum were divided and analyzed using dorsal and ventral sub-regions at each recording site. Five recording depths separated by 400 µm were used in each recording site. For each animal, data from the two most dorsal recording depths were used to characterize the dorsal striatum (Str) (sites 1 and 2) and the two most ventral sites were used similarly to characterize the ventral striatum/nucleus accumbens (NAc) of the rostral-medial recording site (site 1) or ventral Str of the caudal-lateral recording site (site 2). Data from DA-release studies were grouped similarly based on heterogeneous DA innervation of the striatum (Fallon and Moore, 1978b; Lindvall and Bjorklund, 1974). Data from the dorsal and ventral sub-regions of all animals were combined, averaged and analyzed by a two-tailed unpaired *t* test to compare differences at the same recording sub-region between genotypes. Data from the respective dorsal and ventral sub-regions remained separated by recording site.

Histology

After electrochemical recordings, animals were decapitated under deep anesthesia and the brains were dissected for histological studies to confirm electrode placement in the striatum (see Histology). The observed electrode placement in the striatum is shown (see Figure 4.1).

Semi-Quantitative *In Situ* Hybridization Histochemistry

These studies were conducted at the Medical University of South Carolina by Heather Boger, Ph.D. Semi-quantitative *in situ* hybridization of the DA D_2 receptor was performed on *Gdnf*$^{+/-}$ (*n* = 4) and WT mice (*n* = 6) as previously described (Wang and McGinty, 1995). Striatal sections were cryo-sectioned (12 µm) and mounted on Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were pretreated to fix and defat the tissue and block non-specific hybridization. A 1:1 mixture of synthetic cDNA oligodeoxynucleotide probes, complementary to the long form of D2 receptor mRNA ($D2_{244}$; corresponding to bases 728-763) and the short form of D2 receptor mRNA ($D2_{415}$; corresponding to bases 704-739), was labeled using alpha-[35S]-dATP (Amersham Biosciences, Piscataway, NJ, USA) and terminal deoxynucleotidyl transferase (Roche Diagnostics Corporation, Indianapolis, IN, USA). Sections were immersed in 5.0 x 10^5 cpm/20 mL hybridization buffer per section overnight (15 hours) at 37^{o}C in a humid environment. Sections were washed and air dried before being placed into a film cassette with ^{14}C standards (American Radiolabeled Chemicals, St Louis, MO, USA) and Kodak Biomax film (Rochester, NY, USA) for 2 weeks. Quantitation of the mRNA hybridization signals was performed using NIH image 1.62 software as previously described (Wang and McGinty, 1995).

Immunoblotting

These studies were conducted at the Medical University of South Carolina by Heather Boger, Ph.D. Striatal samples (20 µg) from 12-month-old WT and *Gdnf*$^{+/-}$ mice (*n* = 4) were loaded in duplicate and separated on 4-12% NuPAGE Bis Tris gels (Invitrogen, Carlsbad, CA) at 150V for 45 minutes. After separation onto the gel, the gel was transferred via wet transfer onto a nitrocellulose membrane for one hour at 30 V. The membrane was then removed from the cassette and blocked for one hour in 5% non-fat milk in PBS at room temperature and subsequently incubated overnight at room temperature in a DA D_2-receptor primary antibody (1:200, mouse monoclonal, Santa

Cruz Biotechnology, Inc., Santa Cruz, CA, sc-5303). After 24 hours, blots were washed in PBS-T (0.1% Tween-20) and incubated in secondary antibody (1:5000, donkey anti-mouse IgG, Jackson Immunoresearch, West Grove, PA, 715-035-151) in 5% non-fat milk in PBS for 1 hour at room temperature. Blots were then washed in PBS-T and imaged on a Kodak Image Station 4000 (4 exposures, 15 sec each) using Immobilon chemiluminescent reagent (Millipore, Bellerica, MA). For loading control, blots were incubated with anti-actin antibody for 1 hour at room temperature, followed by incubation with secondary antibody and were re-imaged under the same settings. The samples were normalized to actin. Integrated density of the bands was analyzed using AlphaEaseFC (FluorChem 9900) and results were reported as percent of WT.

Determination of Tissue Neurochemical Content by HPLC-EC

Tissues from a separate cohort of mice were used for neurochemical analysis. Tissues were collected at the Medical University of South Carolina by Heather Boger, Ph.D. (see Tissue Preparation and Neurochemical Analysis of Tissues (HPLC-EC)). Mice were anesthetized with isoflurane and euthanized by decapitation. Brains were quickly removed and placed in an ice-cold mouse brain matrix (ASI instruments, Warren, MI). Striatal tissue was collected to reflect the general recording sites from the electrochemical studies. Accordingly, rostral and caudal brain slices were obtained and tissue punches from the dorsal striata were collected from each slice. Tissue analysis by HPLC-EC was conducted at the University of Kentucky by Stewart Surgener. *Gdnf*$^{+/-}$ (*n* = 7) and WT (*n* = 8) mice were compared by a two-tailed unpaired *t* test, keeping rostral and caudal slices separated.

Locomotor Activity

Locomotor activity measures were performed at the Medical University of South Carolina by Heather Boger, Ph.D. Locomotor activity (total distance traveled) was assessed using a Digiscan animal activity monitor system (Omnitech Electronics Model RXYZCM (8); TAO, Columbus, OH, USA) described previously (Halberda*, et al.*, 1997). Animals (*n* = 7) were injected with vehicle (0.9% NaCl, 0.01mL/g body weight, i.p.) and placed in the activity boxes for 1 hour. After 1 hour, mice were injected with the DAT inhibitor, nomifensine (7.5 mg/kg, i.p.), and placed into the activity boxes for an additional 2 hours. Total distance traveled was calculated over 15-minute intervals and averaged for each genotype.

Basal locomotor activity data collected following saline injections were analyzed with a 2 (Genotype) x 4 (Interval) mixed-factor ANOVA with repeated measures on the interval factor. Because of the genotype difference in basal activity, data collected following the nomifensine injections were normalized (expressed as percent change) and analyzed with a 2 (Genotype) x 8 (Interval) ANOVA with repeated measures on the interval factor.

Data Analysis and Statistics

Outliers > 2 standard deviations from the mean were removed from all data. Statistical significance was defined as $p < 0.05$ for all analyses. In reported statistics, *n* represents the number of animals unless otherwise indicated.

Results

Nomifensine-Induced Locomotor Activity

Locomotor activity of 12-month-old *Gdnf*$^{+/-}$ and WT mice was investigated to determine age-related changes in the movement abilities of the *Gdnf*$^{+/-}$ mice and the effects of a catecholamine uptake inhibitor on locomotion in these mice. As seen in Figure 4.3A, the spontaneous locomotor activity following saline (vehicle) injection was diminished in the 12-month-old *Gdnf*$^{+/-}$ mice compared to that of WT mice. A 2 (Genotype) x 4 (Interval) mixed-factor ANOVA, with repeated measures on the interval factor, provided statistical support for reduced motor activity observed for the *Gdnf*$^{+/-}$ mice [Genotype effect: $F_{1,12}= 15.179$, $p < 0.01$].

Following the habituation period, mice were injected with nomifensine – a DAT inhibitor that causes increased locomotor activity (Altar and Marshall, 1988; Hebert and Gerhardt, 1998). Because *Gdnf*$^{+/-}$ mice were less active than WT controls during the habituation period, data generated following the nomifensine injections were expressed as change in motor activity relative to the last 15-minute base interval following saline injection (percent change). Stimulated locomotor activity (Figure 4.3B) showed a greater nomifensine-induced increase in the 12-month-old *Gdnf*$^{+/-}$ mice compared to WT mice. A genotype x interval ANOVA indicated a significant effect of genotype [$F_{7,84} = 3.119$, $p < 0.01$] with *Gdnf*$^{+/-}$ mice demonstrating greater nomifensine-induced locomotor activity during the earlier intervals.

Dopamine Clearance and Uptake Studies

Studies of DA clearance using *in vivo* electrochemical methods were carried out in the striata of *Gdnf*$^{+/-}$ and WT mice to further investigate the functional properties of the DAT *in vivo*. These methods have been shown to reliably determine the relative density of DAT and its kinetic properties in mice and rats (Thomas*, et al.*, 2007). Local application of DA solution was used to obtain DA signals of equivalent amplitude (771 ± 16, mean ± SEM (nM), *n* = 107 signals) for DA-uptake analysis. Genotypes were compared using k_{-1} (sec^{-1}) (Figure 4.5), the first-order rate constant of the decay of the DA signals. *Gdnf*$^{+/-}$ mice and WT mice did not show significant differences in the k_{-1} in the dorsal striatum of either recording site: rostral-medial (site 1) [$p > 0.05$], caudal-lateral (site 2) [$p > 0.05$] (Figure 4.5). However, DA uptake kinetics measured by k_{-1} were significantly increased in the ventral sub-regions of both recording sites of the *Gdnf*$^{+/-}$ mice *versus* WT mice [rostral-medial (site 1): $t_{15} = 2.280$, $^{*}p = 0.0377$; caudal-lateral (site 2): $t_{22} = 2.437$, $^{*}p = 0.0233$] (Figures 4.4; 4.5 A, B). The ventral Str/NAc (recording site 1) of *Gdnf*$^{+/-}$ mice showed a 96% increase in mean k_{-1} compared to WT counterparts [mean ± SEM (sec^{-1}) WT: 0.0023 ± 0.00045, *n* = 12; *Gdnf*$^{+/-}$*:* 0.0045 ± 0.0011, *n* = 5; *n* represents the number of signals]. The analogous ventral striatal sub-region of recording site 2 showed a 100% increase in DA uptake [mean ± SEM (sec^{-1}) WT: 0.0019 ± 0.00016, *n* = 12 *Gdnf*$^{+/-}$*:* 0.0038 ± 0.00076, *n* = 12; *n* represents the number of signals].

A second series of studies compared the amplitudes of the DA signals achieved per amount of DA solution locally applied to investigate surface expression of the DAT within striatal sub-regions. We have previously shown that the amplitude of DA signals per amount of DA applied increases as the surface expression of DAT decreases (Cass and Gerhardt, 1995; Hebert and Gerhardt, 1999). The resulting amplitude per picomole varied according to striatal sub-region and genotype (Figure 4.6; 4.7). Increased DA uptake in the *Gdnf*$^{+/-}$ mice was evident as the resulting amplitude per picomole of applied DA solution was reduced in the *Gdnf*$^{+/-}$ mice. This is evident in both dorsal recording sub-regions in *Gdnf*$^{+/-}$ mice (Figure 4.7). There was a 74% decrease in amplitude per amount of applied DA in dorsal striatum of *Gdnf*$^{+/-}$ mice compared to WT mice [mean ± SEM (nM/pmol) (site 1): WT: 167.7 ± 35.6, *n* = 14; *Gdnf*$^{+/-}$*:* 43.35 ± 11.3, *n* = 10; ($t_{22} = 2.86$, $^{**}p = 0.0091$); *n* represents the number of signals] (Figure 4.7 A). *Gdnf*$^{+/-}$ mice and WT mice did not show significant differences in the amplitude per picomole in the ventral Str/NAc (site 1) [$p > 0.05$] (Figure 4.7 A). Recording site 2 (Figure 4.7 B) showed a 82% decrease in amplitude per picomole in the dorsal striatum of *Gdnf*$^{+/-}$ mice [mean ± SEM

(nM/pmol) (WT: 168.6 ± 29.7, n = 12; *Gdnf*$^{+/-}$: 30.23 ± 7.312, n = 10; (t_{20} = 4.153 ***p = 0.0005); n represents the number of signals] and a 69% decrease in the ventral striatum [mean ± SEM (nM/pmol) WT: 206.2 ± 45.2, n = 12; *Gdnf*$^{+/-}$: 64.39 ± 26.25, n = 10; (t_{20} = 2.575, *p = 0.0181); n represents the number of signals]. Thus a significant decrease in the amplitude per picomole of DA that was locally applied and the increase in k_{-1} indicate increased clearance of exogenously applied DA in *Gdnf*$^{+/-}$ mice.

Studies of Dopamine Release

To determine if *Gdnf*$^{+/-}$ mice exhibited altered DA-release properties in the striatum, we measured *in vivo* DA release following local application of potassium solution (120 mM) (Gerhardt*, et al.*, 1986). This technique has been previously shown to reliably measure neurotransmitter release from terminals present in the striatum (Gerhardt*, et al.*, 1986), which primarily reflects DA release (Gerhardt and Hoffman, 2001; Gerhardt*, et al.*, 1984). The change from baseline was calculated following local application of potassium solution and the average amplitude (μM) was calculated to compare genotypes within striatal sub-regions. There were no significant changes in release amplitude at either striatal sub-region in recording site1 (p > 0.05, both sub-regions) (Figure 4.8 A). In recording site 2 (Figure 4.8 B), both recording regions showed a significant increase in release amplitude. The *Gdnf*$^{+/-}$ exhibited a 236% increase in amplitude versus WT in the dorsal striatum (mean ± SEM (μM) WT: 2.90 ± 0.67, n = 12; *Gdnf*$^{+/-}$: 9.75 ± 1.75 n = 10; t_{20} = 3.908, ***p = 0.0009; n represents the number of signals). Similarly, in the ventral striatum there was a significant increase in amplitude (90%) in the *Gdnf*$^{+/-}$ mice *versus* WT mice [mean ± SEM (μM) WT: 0.74 ± 0.30, n = 9; *Gdnf*$^{+/-}$: 7.6 ± 1.79, n = 8; t_{15}= 4.032, **p = 0.001; n represents the number of signals].

Striatal Dopamine D_2-receptor mRNA and Protein Expression

In a separate cohort of mice, striatal DA D_2-receptor mRNA and protein expression was assessed in WT and *Gdnf*$^{+/-}$ mice to determine a potential connection between altered D_2-receptor expression and DAT activity (Figure 4.9 A). Using *in situ* hybridization histochemistry, striatal DA D_2-receptor mRNA expression was determined. *Gdnf*$^{+/-}$ mice had significantly higher DA D_2-receptor mRNA in the dorsal striatum than WT mice (Figure 4.9 A, B) [t_8 = 3.387, **p = 0.0095, mean ± SEM (percent of WT), *Gdnf*$^{+/-}$: 217.0 ± 29.8 n = 4; WT: 100 ± 20.2, n = 6]. In addition to an increase in striatal DA D_2-receptor mRNA, D_2-receptor protein in the striatum was also increased in 12-

month-old *Gdnf*$^{+/-}$ mice *versus* WT mice (Figure 4.9 B, t_6= 3.192, **p* = 0.0188, mean ± SEM (percent of WT), *Gdnf*$^{+/-}$: 130.3 ± 8.53 *n* = 4; WT: 100 ± 4.21, *n* = 4).

Striatal Tissue Measures of Dopamine and Metabolites and Serotonin and Metabolites by HPLC-EC

To determine if there were concurrent alterations in DA-synthesis or -metabolism, analyses of DA and primary metabolites (DOPAC, HVA) were performed on tissue samples harvested from rostral (site 1: anterior-posterior +1.0) and caudal (site 2: anterior-posterior +0.1) sections of the mouse striatum (*n* = 7-8 per genotype; Table 4.1). The *Gdnf*$^{+/-}$ mice had lower striatal tissue levels of DA *versus* WT mice in both brain sections [site 1: anterior-posterior +1.0, t_{13} = 3.541, ***p* = 0.0036; site 2: anterior-posterior +0.1, t_{13} = 2.281, **p* = 0.0401]. The genotypes did not differ in tissue content of DOPAC or HVA at either set of coordinates. Dopamine turnover was significantly elevated at both recording sites in *Gdnf*$^{+/-}$ mice as indicated by the increase in ratios of HVA:DA [site 1: anterior-posterior +1.0, t_{13} = 3.631, **p* = 0.0268; site 2: anterior-posterior +0.1, t_{13} = 3.812, ***p* = 0.0022] and (DOPAC + HVA):DA [site 1: anterior-posterior +1.0, t_{13}=2.496, **p* = 0.0268; site 2: anterior-posterior +0.1, t_{13} = 4.092, ***p* = 0.0013]. In addition, *Gdnf*$^{+/-}$ mice differed in the DOPAC:DA ratio compared to WT mice in the caudal striatum [site 2, anterior-posterior +0.1, t_{13} = 2.264, **p* = 0.0413]. Thus, *Gdnf*$^{+/-}$ mice demonstrated decreased DA tissue storage, which likely contributed to decreases in DA turnover ratios.

Whole tissue levels of 5-HT and the primary metabolite 5-hydroxyindoleacetic acid (5-HIAA) were also investigated by HPLC-EC from striatal tissues (Table 4.1). The genotypes did not differ in tissue levels of 5-HT or 5-HIAA at either recording site (*p* > 0.05). However, the ratio of 5-HIAA:5-HT in the caudal region of the striatum (site 2) was significantly greater in *Gdnf*$^{+/-}$ mice compared to WT mice [t_{13} = 2.388, **p* = 0.03].

Discussion

These studies reveal dynamic alterations in DA neurotransmission in the striatum of middle-aged *Gdnf*$^{+/-}$ mice. Locomotor experiments indicated lower spontaneous activity in the 12-month-old *Gdnf*$^{+/-}$ mice than in WT mice, confirming previous reports (Boger*, et al.*, 2006). By contrast, nomifensine produced greater relative increases in locomotor activity of the *Gdnf*$^{+/-}$ mice, suggesting alterations in DAT function and/or postsynaptic DA signaling. Behavioral data also demonstrated that mice with a partial

Gdnf deletion are capable of achieving increased locomotor activity comparable to and surpassing WT activity. Electrochemical recordings were conducted to determine if there were presynaptic functional changes in DA uptake mechanisms or the magnitude of evoked DA release. *In vivo* electrochemical recordings of DA uptake indicated a higher rate, k_{-1}, of DA uptake in the ventral striatal sub-regions of *Gdnf*$^{+/-}$ mice compared to WT mice. In addition, *Gdnf*$^{+/-}$ mice showed significant decreases in the amplitude per picomole of DA applied in multiple striatal sub-regions compared to WT mice. Thus, the chronoamperometric studies confirm that *Gdnf*$^{+/-}$ mice have increased high-affinity DA uptake *in vivo,* consistent with an increase in the relative B_{max}. Additionally, DA release was altered in dorsal and ventral striatal recording sub-regions. Assessment of striatal tissue levels of DA and DA metabolites indicated lower DA levels in the *Gdnf*$^{+/-}$ mice, which is consistent with decreased spontaneous locomotor activity and fewer TH-positive neurons in the SNc (Boger*, et al.*, 2006). Taken together, these data demonstrate *in vivo* functional changes in middle-aged *Gdnf*$^{+/-}$ mice that support a role for GDNF in age-related DA-neuron dysfunction (Yurek and Fletcher-Turner, 2001; Yurek*, et al.*, 1998).

Middle-aged *Gdnf*$^{+/-}$ Mice Show Enhanced Behavioral Sensitivity to DAT Inhibition by Nomifensine

The diminished spontaneous activity observed for the 12-month-old *Gdnf*$^{+/-}$ mice corroborates results of a previous study that established a locomotor activity reduction and, furthermore, a positive correlation between reduced motor activity and fewer TH-positive neurons in the SNc (Boger*, et al.*, 2006). Consistent with previous reports, DAT inhibition by nomifensine increased spontaneous motor activity in both genotypes (Altar and Marshall, 1988; Marshall and Altar, 1986). Although the DAT inhibitor elevated activity in both genotypes, the response was increased in the *Gdnf*$^{+/-}$ mice. Importantly, the increased stimulated locomotor activity in *Gdnf*$^{+/-}$ mice suggests that *Gdnf*$^{+/-}$ mice are capable of increased activity i.e. lower motor function that may be affected by GDNF depletion is intact (Oppenheim*, et al.*, 2000). Interestingly, the enhanced response to nomifensine seen for middle-aged *Gdnf*$^{+/-}$ mice in the current study was not observed in other experiments using younger (3-month-old) *Gdnf*$^{+/-}$ mice (Boger*, et al.*, 2007). Consideration of results in these and previous experiments suggests that GDNF depletion and the aging process interact and result in progressive alteration of nigrostriatal function. This interpretation is consistent with the increase in DAT activity

observed in aging (from 3- to 12-month-old) *Gdnf*$^{+/-}$ mice and not WT counterparts (Boger*, et al.*, 2007). Diminished DA uptake is evident in aged rats (Friedemann, 1992; Hebert and Gerhardt, 1999) and is accompanied by an attenuated effect of nomifensine on locomotor behavior (Stanford*, et al.*, 2002) and reductions in DAT density (Hebert*, et al.*, 1999). Considering these previous studies in our model, the increased sensitivity to nomifensine suggests an increase in high-affinity DA uptake by the DAT in the *Gdnf*$^{+/-}$ mice, which was confirmed by the *in vivo* chronoamperometric studies discussed below.

Previous work, showing comparatively weak inhibition of the 5-HT transporter (SERT) by nomifensine (Tuomisto, 1977) and a lack of effect of SERT inhibition on locomotor activity (Hebert and Gerhardt, 1998), support a minor, if any, contribution from serotonergic systems in these studies. Further evidence is provided by striatal tissue analysis for 5-HT and 5-HIAA (see Middle-aged Gdnf+/- Mice Demonstrate Decreased DA Synthesis and increased DA Turnover without Regional Alterations in 5-HT). Regarding potential involvement of serotonergic systems in the clearance studies (discussed below), prior work shows the clearance of locally applied DA in the striatum is unchanged following selective inhibition of SERT (Cass and Gerhardt, 1995). Thus, the described alterations are most likely due to changes in DAT function.

Middle-aged *Gdnf*$^{+/-}$ Mice Show Increased Dopamine Uptake *In Vivo*

Aged rats are reported to have changes in DA-uptake and -release kinetics in the dorsal striatum that are postulated to contribute to age-related motor deficits (Hebert and Gerhardt, 1998). In the current study the increase in the decay constant, k_{-1}, in the ventral striatum (caudal-lateral, site 2) and ventral Str/NAc (rostral-medial, site 1) indicates a more selective effect on mesoaccumbens (A10) DA projections *versus* nigrostriatal (A9) DA projections, which are the primary source of DA in the dorsal striatum (Bentivoglio and Morelli, 2005). We would expect limbic behaviors, particularly those influenced by DAT modulation, to be altered in *Gdnf*$^{+/-}$ mice. Indeed, altered response to reward has been demonstrated in *Gdnf*$^{+/-}$ mice (Airavaara*, et al.*, 2007; Yan*, et al.*, 2007). Increased DA clearance in mesolimbic projections of *Gdnf*$^{+/-}$ mice is also interesting in light of prior findings that GDNF supplementation attenuates behavioral adaptations following administration of addictive drugs (Ghitza*, et al.*, 2009; Messer*, et al.*, 2000). Although reward and reinforcing behaviors are traditionally attributed to mesolimbic DA projections (Di Chiara and Imperato, 1988), mesolimbic DA neurotransmission in the NAc and surrounding ventral striatum may also influence motor

activity (Koob*, et al.*, 1981; Pijnenburg*, et al.*, 1976) – a function historically associated with nigrostriatal DA projections (Kirik*, et al.*, 1998). With consideration of placement variation between animals, striatal heterogeneity (Glynn and Yamamoto, 1989) and the diffuse anatomical boundaries of mesolimbic and nigrostriatal projections, it is unlikely that either of the ventral sub-regions studied reflect mesoaccumbens projections exclusively (Beckstead*, et al.*, 1979; Fallon and Moore, 1978a). We believe DA innervation from the SNc likely contributes to the uptake changes seen in the ventral sub-regions. Indeed, analysis of the amplitude per picomole of DA solution applied indicates uptake changes in both dorsal sub-regions (sites 1 and 2) and the ventral striatum (site 2). Previous work has shown that the positive relationship between DA-signal amplitude from increasing amounts of locally applied DA solution is linear (Cass and Gerhardt, 1995; Hebert and Gerhardt, 1999). The relationship between the amount of locally applied DA and the resulting amplitude is associated with DAT function and was previously shown to reflect an *in vivo* B_{max}, which allows for comparison of the relative number of functional DA-uptake sites (Cass and Gerhardt, 1995; Cass*, et al.*, 1993; Hoffman and Gerhardt, 1998).

The increased striatal DA uptake in *Gdnf*$^{+/-}$ mice provides *in vivo* confirmation of earlier *in vitro* work indicating increased DAT activity in striatal synaptosomes from *Gdnf*$^{+/-}$ mice (~30% increase in DA uptake compared to age-matched WT mice) (Boger*, et al.*, 2007). Because *in vitro* experiments indicated increased DAT activity in tissue from *Gdnf*$^{+/-}$ mice without changes in DAT protein expression, upregulation of DAT expression is unlikely to underlie the current findings. An increase in B_{max} due to increased surface expression of DAT on the plasma membrane of DA terminals and varicosities in the striatum, however, supports the enhanced sensitivity to DAT inhibition by nomifensine seen in the locomotor data as well as chronoamperometric studies. Indeed, increased surface expression of the DAT in *Gdnf*$^{+/-}$ mice would explain the discrepancy between unchanged DAT protein levels and increased DAT activity *in vitro* (Boger*, et al.*, 2007). Future studies should be conducted to determine the surface expression of the DAT in *Gdnf*$^{+/-}$. A possible underlying mechanism regarding DAT surface expression is decreased internalization of the transporter via attenuated activity of protein kinase C (PKC), which has been implicated in molecular control of endocytosis of the DAT (Sorkina*, et al.*, 2005). This possibility requires further investigation.

Altered DA uptake is common to aging studies in rats (Hebert and Gerhardt, 1998; Yurek*, et al.*, 1998) and the *Gdnf*$^{+/-}$ mice. The former involves diminished uptake

while the model examined here demonstrates increased DA uptake with an enhanced response to nomifensine rather than the attenuated response observed in prior studies in aged rats (Hebert and Gerhardt, 1998). Changes in DAT activity have been postulated to function in maintaining DA signaling in lesioned (Hoffman and Gerhardt, 1998; van Horne*, et al.*, 1992) and aged (Hebert and Gerhardt, 1999; Hebert*, et al.*, 1999) animal models of DA dysfunction – modulating signal duration by increasing the amount of DA and/or time synaptic DA is available. We postulate that increased DAT activity observed for *Gdnf*$^{+/-}$ mice is accompanied by relative decreases in tonic levels of synaptic and extracellular DA resulting in decreased postsynaptic DA-receptor activation.

Middle-aged *Gdnf*$^{+/-}$ Mice Show Increased Dopamine Release *In Vivo*

The phasic component of DA release is investigated in the electrochemical potassium-evoked release studies. To our knowledge DA release has not been investigated in middle-aged *Gdnf*$^{+/-}$ mice. Release dynamics are of interest in the context of DA neurotransmission, which has been associated with regulation of GDNF expression (Ohta*, et al.*, 2003). Compensatory mechanisms involving DA release or postsynaptic aspects of DA signaling in *Gdnf*$^{+/-}$ mice may develop to counter GDNF protein reductions. More widely studied than GDNF depletion, GDNF supplementation and DA release have been investigated in numerous animal models (Cass*, et al.*, 2000; Grondin*, et al.*, 2003) including the normal (young) (Hebert*, et al.*, 1996) and aged rat (Hebert and Gerhardt, 1997). When delivered to the primary cell bodies of the SNc, GDNF supplementation caused an increase in evoked DA release in the rat striatum (Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996). We would predict GNDF depletion to have a diminishing effect on DA release, which is not the case in this depletion model (*Gdnf*$^{+/-}$*)* with significant increases in release. Generally, in GNDF-supplementation studies GDNF is administered locally (i.e. to the primary cell bodies of the SNc) in the adult rat. The experimental circumstances of previous experiments make direct comparison to *Gdnf*$^{+/-}$ mice problematic, if not inappropriate, because global brain GDNF depletion (Boger*, et al.*, 2006; Gerlai*, et al.*, 2001) and potential impact on developmental processes (Granholm*, et al.*, 2000; Stromberg*, et al.*, 1993) are not comparable to GDNF treatment to a single brain area in the adult animal.

In the aged rodent, motor deficits have been attributed to presynaptic dysfunction including diminished DA-release and -clearance (Hebert and Gerhardt, 1998; Hebert and Gerhardt, 1999; Yurek*, et al.*, 1998). It has been postulated that DA-release and -uptake

dynamics interact to modulate DA signaling i.e. uptake is reduced in order to prolong DA-receptor activation during diminished release. In *Gdnf*$^{+/-}$ mice, the increased DAT activity may exist to meet the demands of increased phasic release of DA. In this case, the exclusivity of increased DA release to recording site 2 is surprising because uptake is altered in both recording sites. Indeed, the interaction of uptake and release phenomena in *Gdnf*$^{+/-}$ mice is highly speculative and it is unclear if a cause-and-effect relationship exists or to which the release and uptake phenomena may be attributed. Characterization of other (younger) age groups is necessary to make more definitive conclusions about the cause-and-effect relationship between DA-release and -uptake in this model.

Middle-aged *Gdnf*$^{+/-}$ Mice Show Increased Dopamine D_2 Receptor mRNA and Protein

Decreased DA-receptor activation would support diminished spontaneous motor activity in *Gdnf*$^{+/-}$ mice reported in this study and previous work (Boger*, et al.*, 2006). However, here we observed an increase in DA D_2-receptor mRNA and protein in the striatum of *Gdnf*$^{+/-}$ mice (Boger*, et al.*, 2004). We hypothesize that increased DAT activity may cause reduced tonic levels of synaptic DA in *Gdnf*$^{+/-}$ mice and a compensatory increase in DA D_2-receptor expression. Thus, the stimulated motor response to nomifensine is exacerbated in the *Gdnf*$^{+/-}$ mice due to: 1) enhanced sensitivity to nomifensine and 2) increased D_2-receptor activation. Because these assays do not examine surface expression/functional DA-receptors, the discussion should be considered with this caveat. In addition, definitive conclusions about the involvement of the D_2 autoreceptor and potential role in DA-uptake properties cannot be made from the protein and mRNA assays, which reflect mainly postsynaptic D_2 receptors (Levey*, et al.*, 1993). The contribution of D_2 autoreceptors is possible but requires more direct investigation. Inhibition of DA D_2-receptors has been shown to produce a modest (~40%) decrease in DA clearance (amp/pmol) (Cass and Gerhardt, 1994) compared to the ~80% decrease in amp/pmol in the middle-aged *Gdnf*$^{+/-}$ mice. Thus, it is unlikely that changes in D_2-autoreceptor function, alone, cause the observed DA-uptake alterations. Additionally, PKC mediated regulation of DAT membrane trafficking, a potential mechanism proposed previously, has been shown to function independently of DA D_2-receptors (Furman*, et al.*, 2009). Although protein and mRNA levels are significantly increased, profound receptor changes are also unlikely considering the modest decline

(~25%) in tissue DA levels, which do not fulfill required deficits (>90%) for receptor-hypersensitivity phenomena (Hefti*, et al.*, 1980). Future studies should be conducted to further investigate striatal D_2-receptor surface expression and activation as well as tonic extracellular DA levels in the *Gdnf*$^{+/-}$ mice.

Middle-aged *Gdnf*$^{+/-}$ Mice Demonstrate Decreased DA Synthesis and Increased DA Turnover without Regional Alterations in 5-HT

Analyses of DA and metabolites were conducted to determine if synthesis and metabolism of DA were altered in the striatum of *Gdnf*$^{+/-}$ mice. Tissues from rostral and caudal striatal sections (recording sites 1 and 2, respectively) showed decreases in DA tissue levels (average ~25%) that indicate decreased DA synthesis. Decreased DA synthesis is expected because of the reduced number of TH-positive cell bodies in the SN (Boger*, et al.*, 2006). Although there is evidence for decreased striatal TH-staining density (Boger*, et al.*, 2007), it is also possible that the relative activity of TH is reduced due to dephosphorylation – an aspect of TH activity enhanced by GDNF supplementation (Salvatore*, et al.*, 2009; Salvatore*, et al.*, 2004). Given the nature of the global brain depletion of GDNF protein in *Gdnf*$^{+/-}$ mice, both nigral and striatal TH phosphorylation may be affected. The increased ratio of metabolites to DA indicate increased DA metabolism. This is likely due to the decreased levels of DA because DA metabolites, DOPAC and HVA, were not significantly different compared to WT mice.

HPLC-EC quantification of 5-HT and its metabolites were carried out to examine serotonergic changes in *Gdnf*$^{+/-}$ mice. There were no changes in whole tissue 5-HT or metabolite (5-HIAA) levels at either recording site, which supports a minor contribution of serotonergic systems in the described studies. Although the ratio of 5-HIAA:5-HT in the caudal region of the striatum (site 2) was increased in *Gdnf*$^{+/-}$ mice *versus* WT mice [p = 0.03], it is unlikely that serotonergic systems influence electrochemical results given the comparatively low density of 5-HT innervation compared to DA in the striatum (Schenk*, et al.*, 1983) and lack of effect of SERT inhibition on DA uptake parameters (Hoffman and Gerhardt, 1998).

Implications and Explanations of Increased Dopamine Transporter Function

Boger et al., (2007) showed increased susceptibility to methamphetamine-induced toxicity in *Gdnf*$^{+/-}$ mice and attributed the susceptibility to increased DAT activity.

Although the mechanisms of increased DAT activity have not been determined, we would predict increased DAT activity and/or surface expression of the DAT to also cause increased susceptibility to other DA-neuron toxins like MPTP and 6-OHDA, which use the DAT for cellular entry and associated toxicity (Gainetdinov*, et al.*, 1997; Storch*, et al.*, 2004).

In 6-OHDA lesioned rats, high-affinity DA uptake by the DAT is diminished due to a profound loss of striatal DA terminals (Hoffman and Gerhardt, 1998). However, previous work showed decreases in TH-positive fiber density and TH-positive neurons in the SN of *Gdnf*$^{+/-}$ mice (Boger*, et al.*, 2006), making increased functional DA terminals in *Gdnf*$^{+/-}$ mice an unlikely cause of increased DAT activity. In addition, the decrease in striatal DA content (see Middle-aged Gdnf+/- Mice Demonstrate Decreased DA Synthesis and increased DA Turnover without Regional Alterations in 5-HT) supports that an increase in functional DA terminals is unlikely.

Altogether, these data support increased DA-clearance capacity in middle-aged *Gdnf*$^{+/-}$ mice, which may be due to increased DAT activity and/or density of DAT membrane expression as seen in the aged rat (Hebert*, et al.*, 1999) and human brain (Volkow*, et al.*, 1994). It is unclear if the GDNF depletion in this model varies within the striatum because previous characterization studies have focused on the entire striatum, excluding the NAc (Boger*, et al.*, 2006). A differential effect of GDNF on DA terminals in the striatal sub-regions due to differential spatial expression of GDNF in this model could contribute to the current findings. These results require further investigation to determine the definitive mechanism(s) that may underlie the apparent uptake changes.

Conclusions

Motor and pathological manifestations associated with the *Gdnf*$^{+/-}$ mice become apparent later in life and progress gradually (Boger*, et al.*, 2006). This progressive time course contrasts with other DA-lesion models where the onset of neurodegeneration is comparatively rapid and severe (Ichitani*, et al.*, 1994; Kirik*, et al.*, 1998; Lee*, et al.*, 1996; Sauer and Oertel, 1994) and deficits may recover with time (Yuan*, et al.*, 2005). The *Gdnf*$^{+/-}$ mouse is a novel model of DA dysfunction that demonstrates some of the subtle aspects of human DA-neuron dysfunction seen in aging. The regulation of GDNF has yet to be clearly defined for aging and DA-neuron dysfunction in general. There are contradictory results regarding the regulation of GDNF in DA-neuron dysfunction and in age-associated movement disorders like PD. It has been suggested that GDNF isoforms

may be differentially expressed in PD with an increase in isoform I (Backman*, et al.*, 2006). By contrast, decreases in GDNF levels have been reported in the SN of PD patients (Jenner and Olanow, 1998). In animal models of DA dysfunction, both increased (Nakajima*, et al.*, 2001) and unchanged levels of striatal GDNF (Smith*, et al.*, 2003) have been reported following 6-OHDA administration. Our studies indicate changes in DA-neurochemistry, -uptake and -release accompanied by behavioral deficits using a model of chronic and partial genetic reductions of GDNF that appears to manifest age-related changes.

Taken together, these studies support a unique role for GDNF in the development, function and maintenance of DA neurons. Further studies are necessary to determine the specific mechanisms that underlie the apparent neurochemical and kinetic changes in DA regulation as well as the widespread implications of these changes.

Portions of this chapter have been published in the manuscript:

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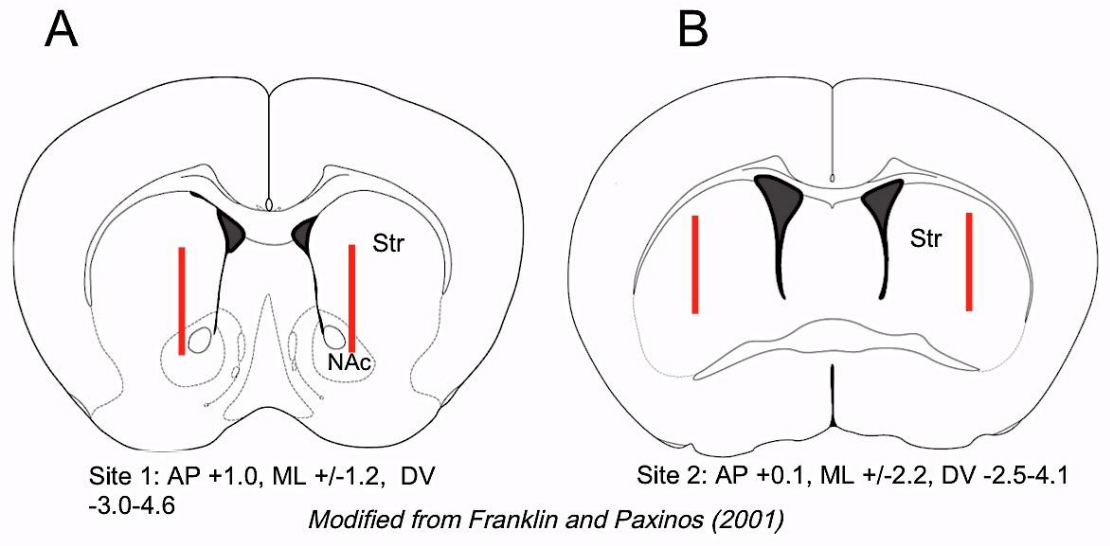

Figure 4.1 Representation of Microelectrode Placement in the Mouse Brain
The diagrams show the approximate location of the microelectrode at (A) site 1 and (B) site 2 (coordinates relative to *bregma* (mm)). Bilateral recordings were conducted for DA-uptake and -release studies. The microelectrode was lowered using increments of 400 µm through the dorsal-ventral axis of the striatum.

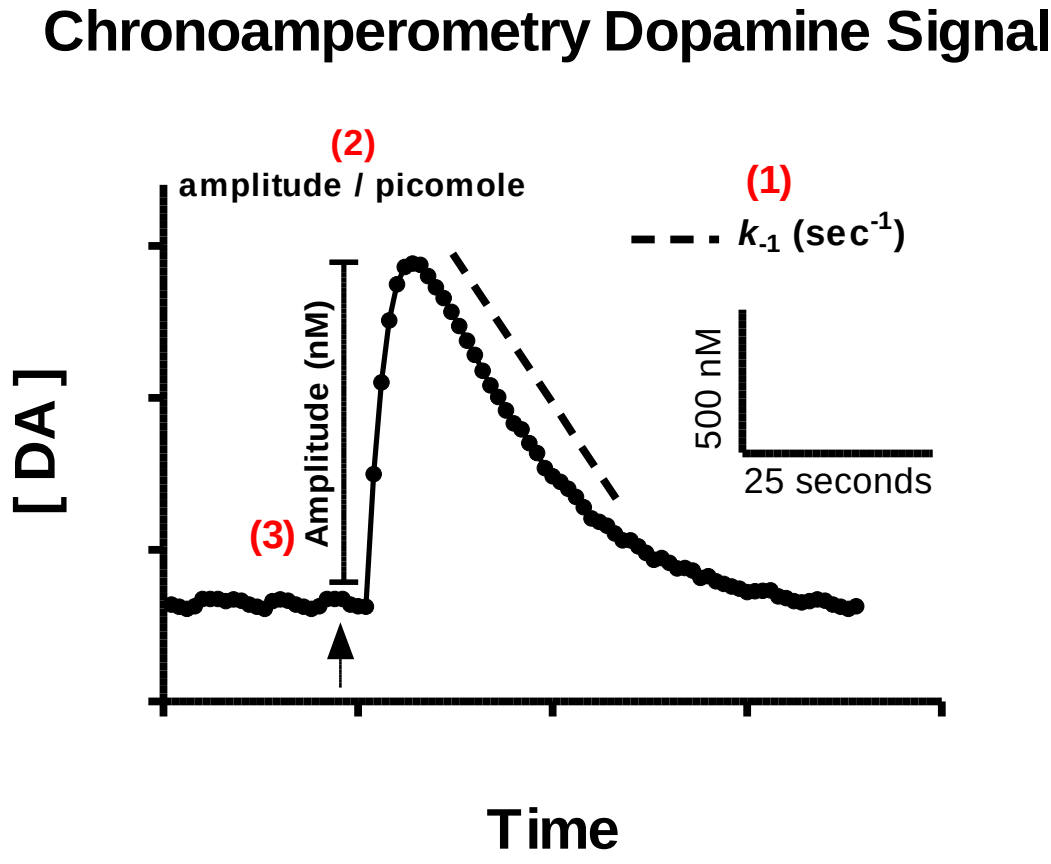

Figure 4.2 Analysis parameters for *In Vivo* Electrochemical Dopamine Signals
The current measured by the microelectrode was converted to concentration (shown) by dividing the measured current (nA) by the calibration slope (nA/μM) determined for each microelectrode prior to use (inset: axes units). Following local application of DA solution (100 μM, indicated by arrow) in the uptake studies, the amplitude was calculated as change from a stable baseline. The first order exponential decay constant, (1) k_{-1} (sec^{-1}), was calculated from the decay portion of the DA signal fitted to the slope of the linear regression of the natural log transformation of the data. By dividing the resulting DA signal amplitude by the amount (picomole) of DA solution applied, the (2) amplitude per picomole was calculated. For DA-release studies, DA signals resulting from local application of high-potassium solution (120 mM, arrow) were analyzed by calculating the (3) maximum amplitude (nM) as the concentration change from baseline.

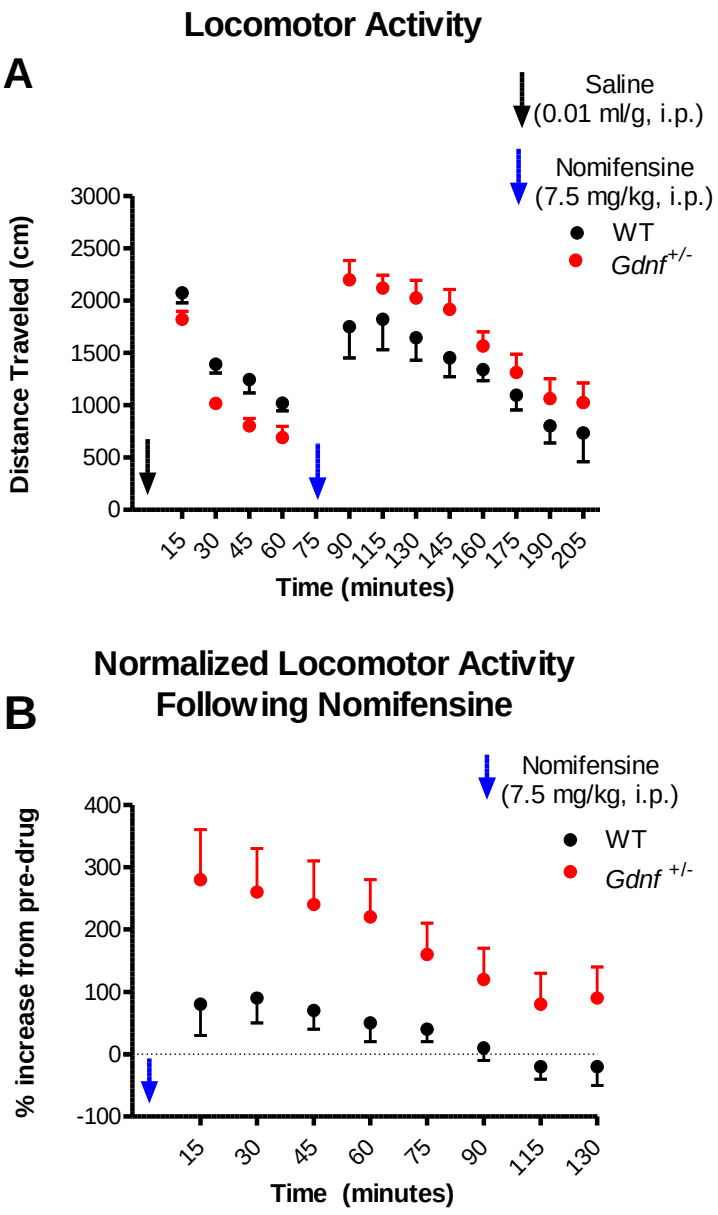

Figure 4.3. Spontaneous and Nomifensine-induced Locomotor Activity in 12-month-old *Gdnf*$^{+/-}$ and WT Mice

Saline-injected *Gdnf*$^{+/-}$ mice showed a decrease in total distance traveled compared to WT mice (A) [$F_{1,12}$ = 15.179, $^{**}p < 0.01$]. *Gdnf*$^{+/-}$ mice showed greater nomifensine-induced locomotor activity compared to nomifensine-treated WT mice (B) [$F_{7,84}$ = 3.119, $^{**}p < 0.01$]. Data are shown as mean ± SEM and were analyzed by a 2 (Genotype) x 4 (Interval) mixed-factor ANOVA with repeated measures on the interval factor (A) and 2 (Genotype) x 8 (Interval) mixed-factor repeated-measures ANOVA.

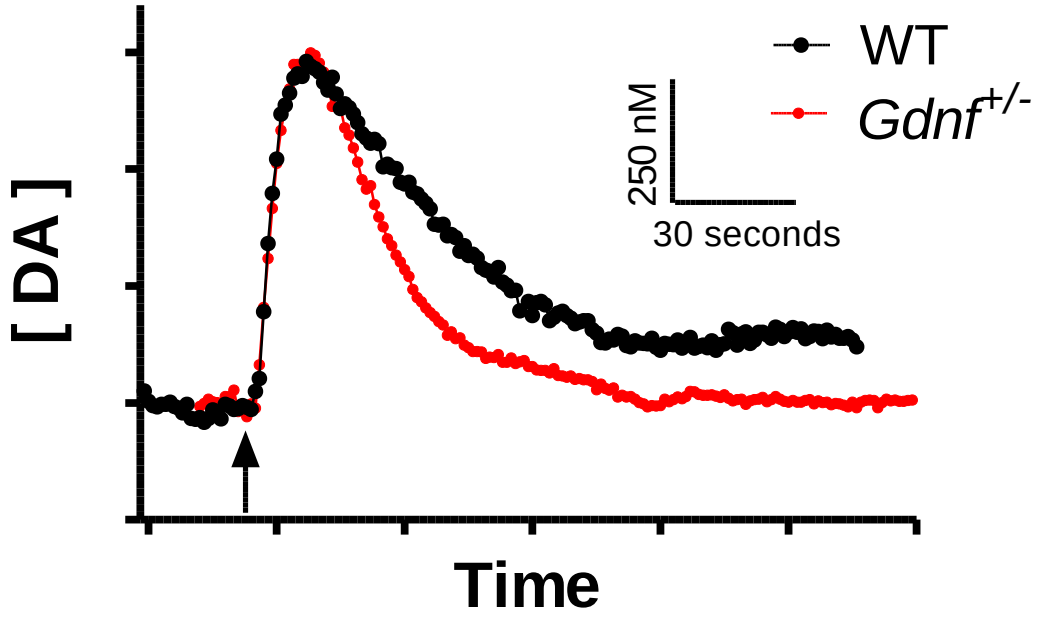

Figure 4.4 Representative Trace of Dopamine Uptake in *Gdnf*$^{+/-}$ Mice
A DA solution (100 μM) was locally applied (arrow) in the mouse striatum to obtain DA signals of equivalent amplitude (inset: axes units). For amplitude-matched DA signals, uptake (quantified as k_{-1}) was increased in *Gdnf*$^{+/-}$ mice – demonstrated by the increase in slope of the decay portion of the signal.

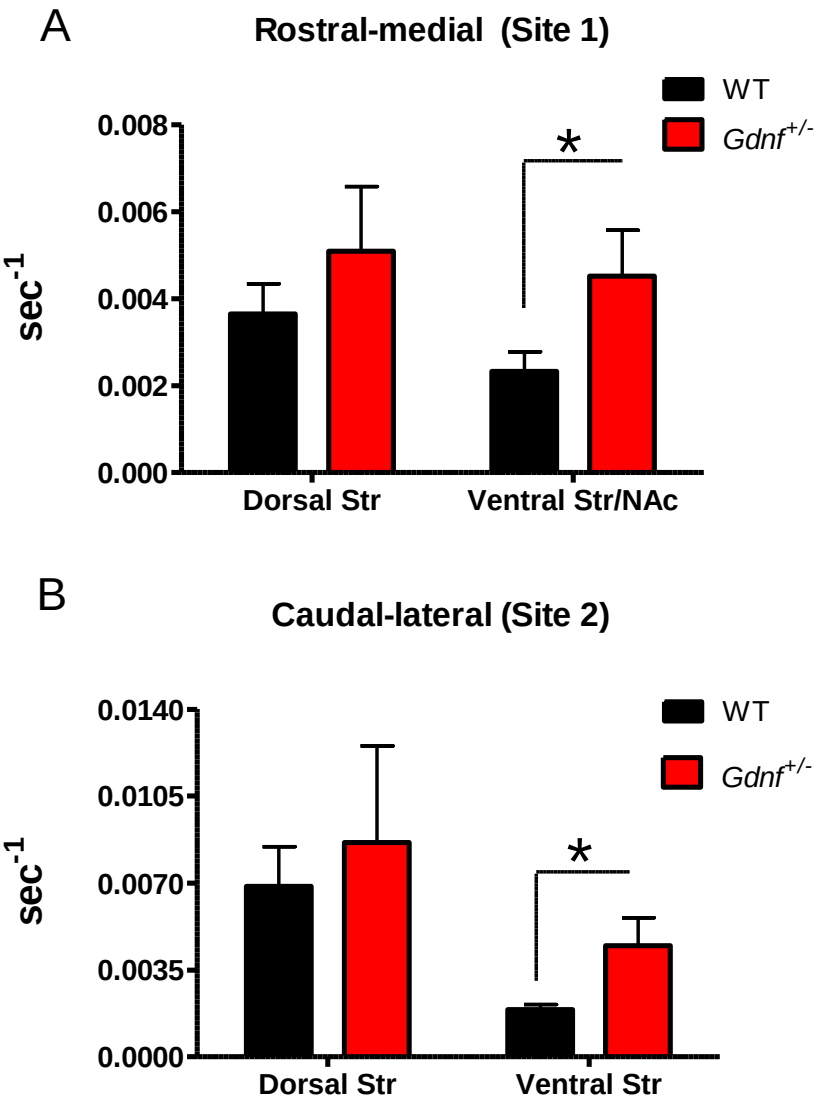

Figure 4.5. Average k_{-1} (sec^{-1}) from Dorsal and Ventral Striatal Recording Sub-regions of *Gdnf*$^{+/-}$ and WT Mice

Local application of DA solution was used to obtain DA signals within an amplitude range, 500-1000 nM, for uptake analysis. The k_{-1} was analyzed to compare *Gdnf*$^{+/-}$ and WT mice. The k_{-1} was not significantly changed between genotypes in the dorsal striatum of the rostral-medial recording site 1 or caudal-lateral recording site 2. The k_{-1} did show genotype differences exclusive to the ventral Str/NAc of the rostral-medial recording site 1 [*p = 0.0377] (A) and ventral striatum of the caudal-lateral recording site 2 [*p = 0.0233] (B). There were significant increases in DA uptake in *Gdnf*$^{+/-}$ mice. Data shown as mean ± SEM and were analyzed by a two-tailed unpaired *t* test comparing genotypes.

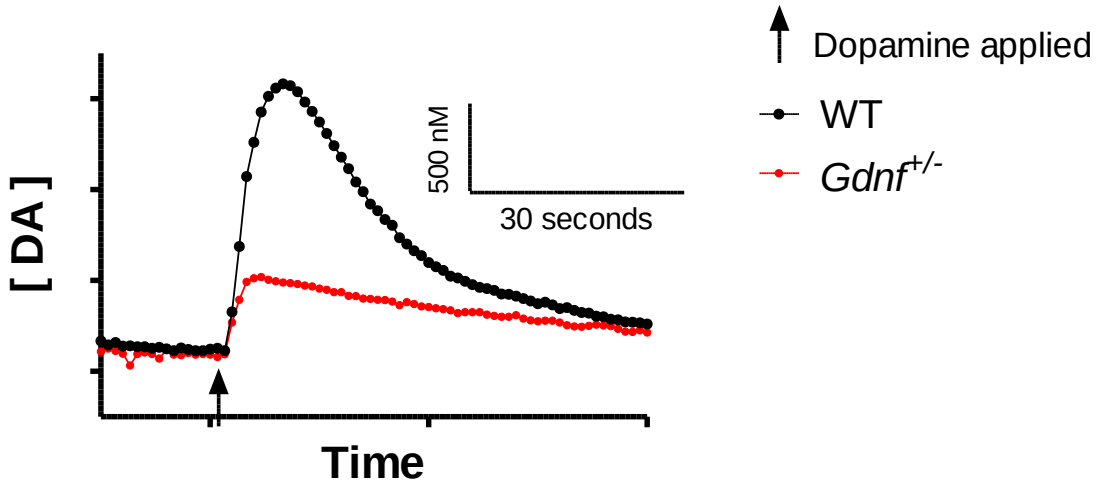

Figure 4.6 Representative Trace of Dopamine Signal Amplitude (for equivalent amount of DA applied) in *Gdnf*$^{+/-}$ Mice

A DA solution (100 µM) was locally applied (arrow) in the mouse striatum. The resulting amplitude (nM) was divided by the amount of DA applied (picomole) to calculate the amplitude per picomole (inset: axes units). The amplitude for an equivalent amount of DA applied was signficantly decreased in *Gdnf*$^{+/-}$ mice.

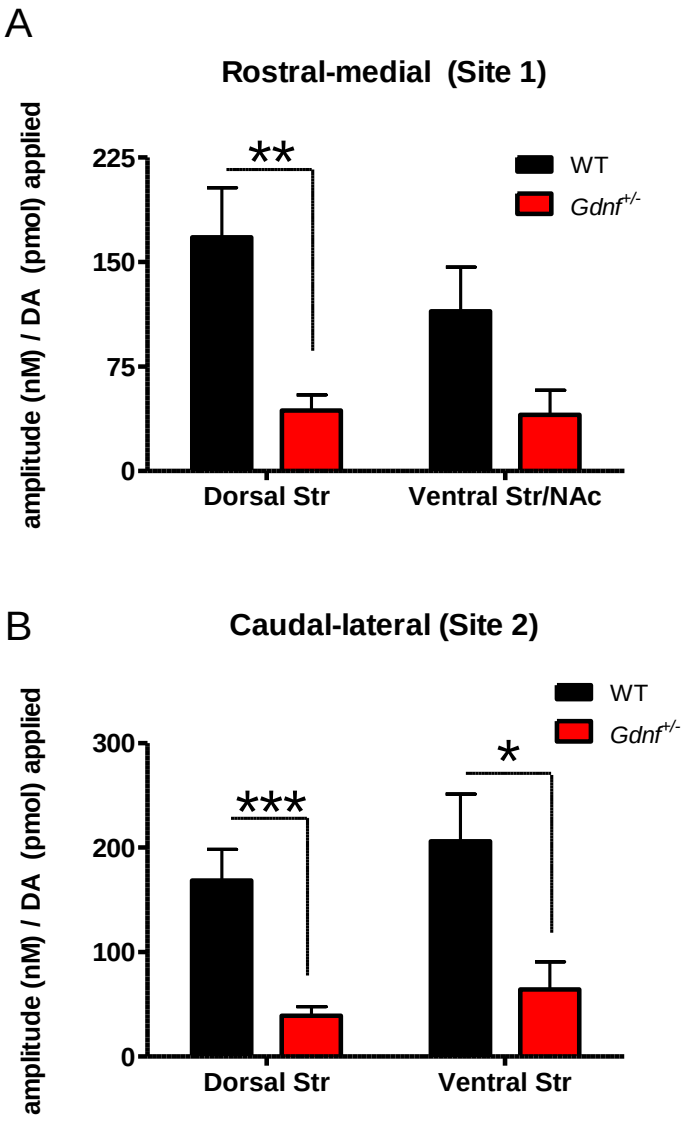

Figure 4.7 Amplitude per Amount of Locally Applied Dopamine in *Gdnf*$^{+/-}$ Mice
The volume of solution was controlled in order to obtain signals of amplitudes ranging from 500-1000 nM for uptake analysis. The resulting amplitude per picomole was analyzed to compare *Gdnf*$^{+/-}$ and WT mice. All striatal sub-regions showed a decrease in the average amplitude per picomole applied in the *Gdnf*$^{+/-}$ mice compared to WT mice. The amplitude per picomole was not significantly different between genotypes in the ventral Str/NAc of the rostral-medial recording site 1 (A). The dorsal striatum, however, showed a significant difference between genotypes [$^{**}p = 0.0091$]. Both recording sub-regions of recording site 2 (B) showed a significant difference in amplitude per picomole [$^{***}p = 0.0005$] (dorsal striatum) and [$^{*}p = 0.0181$] (ventral striatum). Data are shown as mean ± SEM and were analyzed by a two-tailed unpaired *t* test comparing genotypes.

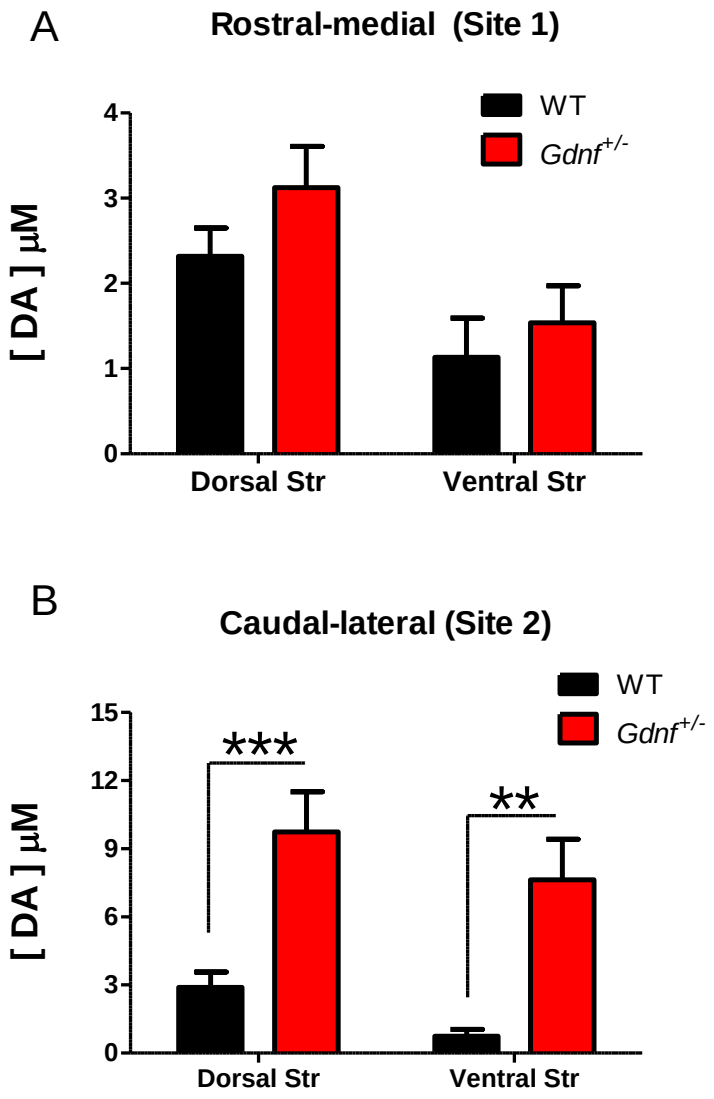

Figure 4.8 Average Amplitude of Evoked-Dopamine Signals from *Gdnf*$^{+/-}$ Mice
A potassium chloride solution (120 mM) was applied locally (75-150 nL) in order to measure DA release in the striatum of *Gdnf*$^{+/-}$ mice. The resulting amplitude from striatal sub-regions was averaged and analyzed to compare genotypes. Neither the dorsal nor ventral striatum/NAc showed significant differences in DA release in the rostral-medial recording site 1 (A). Both striatal sub-regions showed significant differences in DA release in the caudal-lateral recording site 2 (B). *Gdnf*$^{+/-}$ mice demonstrated increased DA release compared to WT mice in the dorsal [***p = 0.0009] and ventral striatum [**p = 0.0011]. Data are shown as mean ± SEM and were analyzed by a two-tailed unpaired *t* test comparing genotypes.

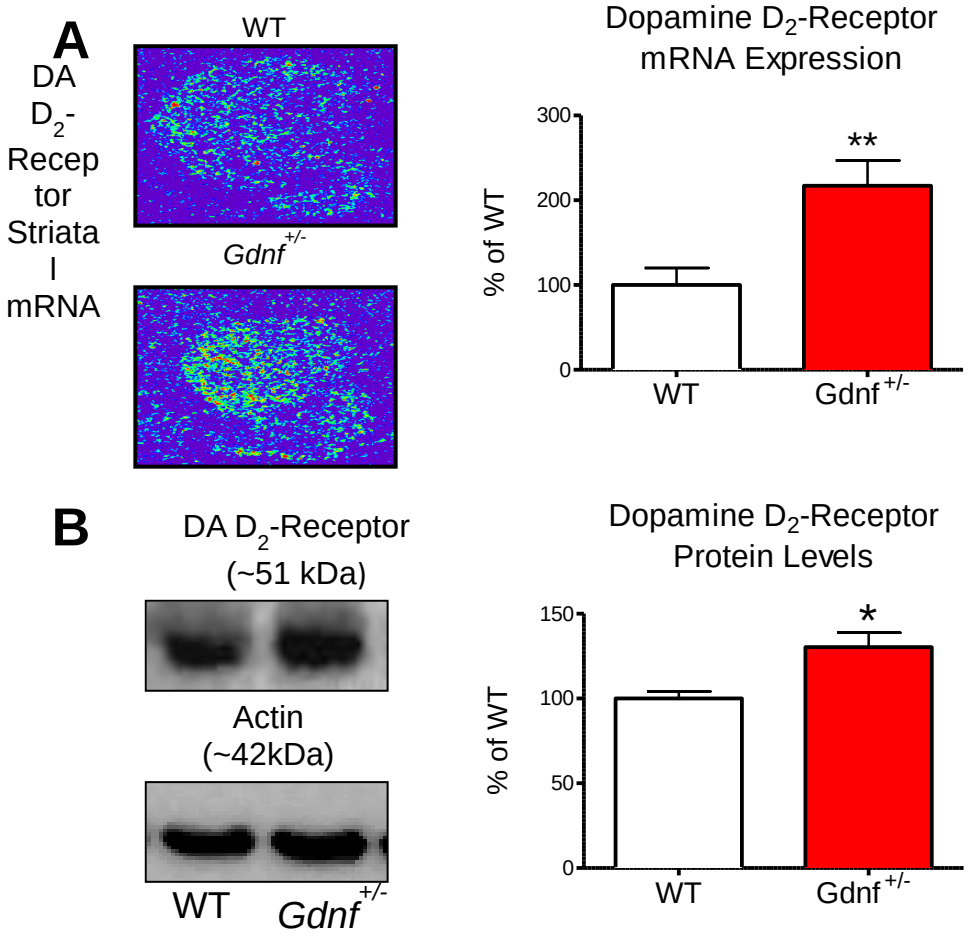

Figure 4.9. Striatal Dopamine D_2-Receptor mRNA and Protein Expression in *Gdnf*$^{+/-}$ Mice *In situ* hybridization immunohistochemical detection indicated an increase in DA D_2-receptor mRNA in the dorsal striatum of *Gdnf*$^{+/-}$ mice compared to WT (A) [**p* = 0.0095]. Protein levels of the DA D_2-receptor (B) were also significantly increased in the dorsal striatum of *Gdnf*$^{+/-}$ mice compared to WT mice [**p* = 0.0188]. Data are shown as mean ± SEM and were analyzed by a two-tailed unpaired *t* test comparing genotypes

Table 4.1 Tissue Neurochemical Content in 12-month-old *Gdnf*$^{+/-}$ Mice

Genotype	Striatal Region	DA	DOPAC	HVA	DOPAC:DA x10 ^{3}	HVA:DA x10 ^{3}	(DOPAC+HVA):DA x10 ^{3}	5-HT	5-HIAA	5-HIAA:5-HT x10 ^{3}
WT	AP: +1.0	19509 ± 399	3198 ± 207	2087 ± 82	164.4 ± 3.83	107.2 ± 1.6	271.6 ± 4.9	874 ± 10	590 ± 6	679.9 ± 10
Gdnf$^{+/-}$	AP: +1.0	15748 ± 1044** $t(13) = 3.54$, $p = 0.0036$	2907 ± 250	2050 ± 97	183.9 ± 7.36	131.6± 5.0** $t(13) = 3.631$, $p = 0.003$	315.5 ± 9.9* $t(13)=2.496$, $p = 0.0268$	854 ± 51	635 ± 19	757 ± 43
WT	AP: +0.1	8656 ± 715	1795 ± 133	1359 ± 70	210.1 ± 3.52	162.1 ± 4.1	372.1 ± 6.9	903 ± 23	653 ± 17	726 ± 8.9
Gdnf$^{+/-}$	AP: +0.1	6027 ± 924* $t(13) = 2.281$, $p = 0.0401$	1487 ± 244	1297 ± 162	244.0 ± 11.3* $t(13) = 2.264$, $p = 0.0413$	222.0 ± 10.3** $t(13) = 3.812$, $p = 0.0022$	466.0 ± 10.3** $t(13) = 4.092$, $p = 0.0013$	867 ± 53	755 ± 56	879 ± 53* $t(13) = 2.388$, $p = 0.03$

Data shown as mean ± SEM (ng/g wet weight of tissue). Analyzed by a two-tailed unpaired *t* test comparing genotypes.

Chapter Five: GDNF Replacement in *Gdnf*$^{+/-}$ Transgenic Mice

Introduction

The use of trophic factor supplementation, particularly in the context of PD, has been widely investigated with promising findings of enhanced neuron function and behavioral measures (Peterson and Nutt, 2008). In particular, GDNF has shown restorative effects in numerous animal models exhibiting DA-neuron dysfunction including the aged and 6-OHDA lesioned rat (Hebert and Gerhardt, 1997; Hoffer*, et al.*, 1994) and the aged and MPTP-lesioned non-human primate (Grondin*, et al.*, 2003; Grondin*, et al.*, 2002). GDNF has also demonstrated protective effects from 6-OHDA- and MPTP-induced cytotoxicity in rodents (Kearns*, et al.*, 1997; Tomac*, et al.*, 1995). GDNF is a target derived neurotrophic factor that is expressed at highest levels in the developing striatum with declining expression levels in adulthood (Stromberg*, et al.*, 1993). In light of the restorative- and protective-effects of GDNF on DA neurons and the prominent role of GDNF in development (Granholm*, et al.*, 2000), the effects of a chronic GDNF depletion have been investigated using GDNF heterozygous mice (*Gdnf*$^{+/-}$), which have decreased GDNF protein expression in the brain (Boger*, et al.*, 2006; Pichel*, et al.*, 1996).

Gdnf$^{+/-}$ heterozygous mice display a unique aging phenotype with locomotor deficiencies, decreases in TH-positive staining in the SN and functional changes in DA-release and -uptake properties in the striatum (Boger*, et al.*, 2006; Littrell*, et al.*, 2010) (see also Chapter Four: Dynamic Changes in Nigrostriatal Dopamine-Neuron Function in Gdnf+/- Mice – A Novel Model of Nigrostriatal Dysfunction). The decline in motor activity is of interest due to the modest reduction (~25%) in DA levels seen in *Gdnf*$^{+/-}$ mice (Littrell*, et al.*, 2010) – far below the typical threshold seen upon emergence of motor deficits in PD (Bernheimer*, et al.*, 1973) and animal models of PD (Hudson*, et al.*, 1993; Kirik*, et al.*, 1998). Functional and behavioral abnormalities in the absence of profound DA-neuron reductions, seen in middle-aged *Gdnf*$^{+/-}$ mice, are also demonstrated with aging in animal models (Grondin*, et al.*, 2003; Hebert and Gerhardt, 1998; Hebert and Gerhardt, 1999) and aging in humans (Bennett*, et al.*, 1996).

Because of indications for enhanced DA-neuron function and parallel behavioral improvements after GDNF treatment in aged animals (Grondin*, et al.*, 2003; Hebert and Gerhardt, 1997), neurochemical function and behavioral measures were investigated after GDNF treatment in *Gdnf*$^{+/-}$ mice. GDNF replacement in a chronic GDNF depletion

model was investigated to test the hypothesis that age-related DA-neuron deficiencies and related behavioral impairments are reduced by GDNF treatment in middle-aged (12-month-old) *Gdnf*$^{+/-}$ mice. The following questions were investigated: 1) Does GDNF treatment affect spontaneous or stimulated locomotor activity? 2) Does GDNF treatment restore the number of DA neurons/DA-neuron fiber density in the SN? 3) Are there changes in DA-neuron functional measures after GDNF treatment?

Materials and Methods

Reagents

Recombinant methionyl human GDNF (Amgen, Thousand Oaks, CA, USA), expressed in Escherichia coli as previously described (Lin, et al., 1993), was used.

Delivery of GDNF

GDNF was dissolved (5 µg/µL) in sterile (0.22 µm filtered) citrate buffer (10 mM sodium citrate, 150 mM NaCl, pH = 5) as described previously (Hebert*, et al.*, 1996).

See Intracranial Drug Delivery - Survival Surgical Procedures for general infusion procedures. *Gdnf*$^{+/-}$ mice (12-month-old) (*n* = 13), age-matched WT littermate mice (*n* = 9) and age-matched (non-transgenic/non-littermate) C57/Bl6 mice (*n* = 7) were treated with 10 µg of GDNF (bilaterally) or equivalent volume of citrate buffer (vehicle) to the striata. The dose was selected based on studies in rodents (Hebert*, et al.*, 1996; Hudson*, et al.*, 1995). Stereotaxic coordinates were (from *bregma* (mm) (bilaterally)): anterior-posterior: +1.0, medial-lateral: +/-1.5, dorsal-ventral: -3.0 (Franklin and Paxinos, 2001; Kirik*, et al.*, 2004). GDNF and vehicle treatments were administered bilaterally at a rate of 0.2 µL/min for 10 minutes. There was significant attrition due to anesthetic intolerance prior to and during electrochemical recording in all treatment groups and genotypes. Thus, some of the treated animals were not viable for use in electrochemical recordings and brain tissue neurochemical analysis. The resulting sample size is indicated in statistics sections.

Locomotor Activity

Animals were monitored for locomotor activity using methods modified from those described previously (see Locomotor Activity). Animals were tested for spontaneous motor activity prior to treatment with vehicle or GDNF and randomly

assigned to treatment groups. Pre-treatment locomotor activity was analyzed using a two-tailed unpaired *t* test. Spontaneous motor activity was determined weekly (1-4 weeks after treatment) using the total distance traveled over the 1-hour period. At the 4-week time point, animals were injected with saline (0.9% NaCl, 0.01mL/g body weight, i.p.) before measuring spontaneous locomotor activity. Spontaneous locomotor activity was analyzed using a 2 (genotype) x 2 (treatment) x 4 (intervals) mixed-factor ANOVA with repeated measures on the interval factor.

Stimulated motor activity was measured (4 weeks after treatment only) using the DAT inhibitor nomifensine (7.5 mg/kg i.p.). Stimulated motor activity was analyzed using a genotype x treatment 1 (GDNF/vehicle) x treatment 2 (nomifensine/saline) three-way ANOVA.

Electrochemical Recordings

Gdnf$^{+/-}$ (12-month-old) and age-matched WT littermate mice were prepared for electrochemical recordings as described (see In Vivo Electrochemical Recording and Microdialysis – Non-Survival Surgical Procedures). Experiments were designed to reproduce previous recordings methods using stereotaxic coordinates described (Figure 4.1) (see also Electrochemical Recordings). To determine DA-release characteristics after GDNF treatment, evoked DA release was investigated (bilaterally) using local application of a high-potassium solution. Electrodes used in these studies were selective for DA (*versus* ascorbate (≥ 70:1)), exhibited linear responses to DA (R^2 ≥ 0.99) and had average detection limits (LOD) of 26.4 ± 5.53 (mean ± SEM (nM), *n* = 31 (electrodes)). Signals used for analysis exhibited reduction:oxidation ratios of 0.75 ± 0.014, mean ± SEM, *n* = 172 (signals) – consistent with the electrochemical profile for DA (Gerhardt and Hoffman, 2001; Gerhardt*, et al.*, 1986).

Data were analyzed using the two most dorsal and two most ventral recording depths from each recording site to determine release characteristics (amplitude change from baseline (Δ, μM)) for striatal sub-regions (Figure 4.1, see also Electrochemical Recordings, Analysis of Electrochemical Recordings).

Determination of Tissue Neurochemical Content by HPLC-EC

Tissues were obtained from the striata using modifications from methods described previously (see Tissue Preparation and Neurochemical Analysis of Tissues (HPLC-EC)). Mice were decapitated while under deep urethane anesthesia. Rostral and

caudal brain slices were chosen to reflect the general recording sites. Brain slices were further sectioned into dorsal and ventral sections based on the heterogeneous functional characteristics (see Dopamine Clearance and Uptake Studies). Analyses of tissue neurochemical content were performed by Wayne Cass, Ph.D.

Substantia Nigra Immunohistochemistry (TH-ir and GDNF-ir)

Immunohistochemistry was performed at the Medical University of South Carolina by Heather Boger, Ph.D as described previously (Boger*, et al.*, 2006). After electrochemical recordings, mice were euthanized while under deep anesthesia and brains were dissected. Brains were stored in 4% paraformaldehyde at 4° C for 24 hours and transferred to 30% sucrose until cryo-sectioning (45 µm; Microm, Zeiss, Thornwood, NY). A polyclonal TH antibody (TH; 1:5000; Pel-Freeze Inc., Roger, AZ) and a polyclonal GDNF antibody (1:100; Santa Cruz Biotechnology, Inc.) were used for free-floating immunohistochemistry on the SN – using every 3rd section for TH and every 6th section for GDNF. Peroxidase activity was quenched by treating sections with H_2O_2, methanol and 0.01 M tris buffered saline (TBS) (ratio of 1:2:7; pH = 7.6) for 15 minutes followed by permeabilization in TBS with 0.25% TritonX-100 (TBST) and treatment with sodium-m-peroxidate (0.1M, in TBS; 20 minutes). Non-specific binding was controlled by incubation at room temperature in 10% normal goat serum for 30 minutes. Sections were incubated with the primary antibody for 24 hours (TBST with 3% NGS), washed with TBST and then incubated with the secondary antibody (1:200, Vector Labs, Burlingame, CA) and avidin-biotin complex (ABC kit, Vector Labs). The reaction was developed using 3′3′-diaminobenzidine (0.05% of 3% H_2O_2) using 2.5% nickel ammonium sulfate to enhance the reaction. Sections were mounted on glass slides and cover-slipped.

Unbiased Stereological Cell Counts

Stereological cell counts were performed at the Medical University of South Carolina by Heather Boger, Ph.D. Quantitative estimates of total numbers of TH-immunoreactive (TH-ir) neurons in SN were determined by unbiased stereological cell counts from serial sections through the midbrain. Methods described previously were followed (Gundersen*, et al.*, 1988; West, 1993). The optical fractionator system used in the present study was Stereo Investigator stereological software (MicroBrightfield, Colchester, VT) coupled to a Prior H128 computer-controlled *x-y-z* motorized stage.

Outline contours were drawn at low magnification (10x) and the outlined region measured with a systematic random design of disector-counting frames. The counting frame area was 2500 μm^2 and the sampling grid area was 100 x 100 µm (Boger*, et al.*, 2006). The counting brick was approximately 20 µm thick after excluding the upper and lower guard zones (2.5 µm each). The slides were coded and TH-ir cells were counted using a 40x objective lens with a 1.4 numerical aperture. The selection of the first section from the SN in the rostral position was random and every 3rd section was counted – rendering a systematic random design. The total number of neurons was calculated. Outline contours drawn for SN cell counts included areas containing predominately TH-labeled dopaminergic neurons (SNc and lateralis). The SNr – containing mostly TH fibers – was excluded. The cell size was measured on every 3rd section through the SN in all 24 groups on the same sections used for stereological assessment (randomly measuring at least 150 neurons per brain across all stereological sections) according to previous protocols (Boger*, et al.*, 2006; Boger*, et al.*, 2007; Zaman*, et al.*, 2008).

GDNF Protein Quantification (ELISA)

GDNF protein quantification was conducted at the Medical University of South Carolina by Heather Boger, Ph.D. Protein levels were assessed using a commercially available assay kit from Promega (Madison, WI) and a standard protocol (Albeck, et al., 2003). Animals used in electrochemical recordings were euthanized (under deep urethane anesthesia) and brains were dissected. The overlying cortex was peeled away from coronal sections and the entire striatum was dissected including the medial and lateral striatum but excluding the nucleus accumbens. Flat-bottom plates were coated with the corresponding capture antibody, which binds the soluble captured neurotrophin. The captured neurotrophin was bound by a second specific antibody, which was detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. Unbound conjugates were removed by subsequent wash steps according to Promega protocols. After an incubation period with chromagenic substrate, color change was measured in an ELISA plate reader at 450 nm. According to the assay kit the quantification range for GDNF was 7.8–500 pg/mL and cross reactivity with other trophic proteins ranged < 2–3%. Protein levels of GDNF were expressed as pg/mg wet weight of tissue.

Data Analysis and Statistics

Outliers > 2 standard deviations from the mean were removed from all data. Statistical significance was defined as $p < 0.05$ for all analyses. In reported statistics, *n* represents the number of animals unless indicated otherwise.

Results

GDNF Protein Levels

To quantify effective increases in GDNF protein after striatal treatment, ELISA was used to determine protein levels in the striatum of GDNF- and vehicle-treated *Gdnf*$^{+/-}$ and WT mice ($n = 4$) (Figure 5.1 B). There was a significant effect of GDNF treatment [$F_{3,12} = 17.46$, $p = 0.0001$] determined by one-way ANOVA. Bonferroni *post hoc* tests show vehicle-treated *Gdnf*$^{+/-}$ and WT mice had significant differences in protein levels [mean ± SEM (pg/mg wet tissue weight), WT: 10.7 ± 0.60, *Gdnf*$^{+/-}$*:* 6.8 ± 0.66, $^{**}p < 0.01$]. There was also a significant difference in protein levels after treatment in *Gdnf*$^{+/-}$ mice [mean ± SEM (pg/mg wet tissue weight), vehicle: 6.8 ± 0.66, GDNF: 9.5 ± 0.39, $^{*}p < 0.05$] without significant difference in protein between vehicle- and GDNF-treated WT mice [$p > 0.05$]. GDNF treatment in *Gdnf*$^{+/-}$ mice restored striatal GDNF levels to those detected in WT mice [$p > 0.05$ *versus* WT (vehicle)]. WT and *Gdnf*$^{+/-}$ mice treated with GDNF had significantly different protein levels [mean ± SEM (pg/mg wet tissue weight, WT: 12.3 ± 0.52, *Gdnf*$^{+/-}$: 9.5 ± 0.39 $^{*}p < 0.05$] as did GDNF-treated WT and vehicle-treated *Gdnf*$^{+/-}$ mice [$^{***}p < 0.001$].

Immunohistochemistry was used to determine GDNF levels in the SN after striatal GDNF treatment (Figure 5.1 A). Qualitative treatment of the results showed an increase in staining intensity after treatment with GDNF. Both genotypes exhibited comparable staining levels after GDNF treatment. These data suggest that striatal GDNF administration increased the supply of GDNF in the SN – indicating retrograde transport of GDNF may occur.

Locomotor Activity

As we have shown previously (Littrell*, et al.*, 2010), spontaneous locomotor activity in *Gdnf*$^{+/-}$ mice (pre-treatment) is decreased ($^{**}p = 0.0022$; $t_{20} = 3.515$, ($n = 8$)) (Figure 5.2). Spontaneous locomotor activity was measured in mice treated with GDNF (n = WT: 5 *Gdnf*$^{+/-}$*:* 7) or vehicle (n = WT: 4-5 *Gdnf*$^{+/-}$*:* 7) and analyzed using a 2

(genotype) x 2 (treatment) x 4 (intervals) mixed-factor ANOVA with repeated measures on the interval. Analysis shows a significant genotype x treatment interaction ($F_{1,79}$= 5.114, p = 0.0265) as well as a significant treatment x time interaction ($F_{3,79}$ = 9.255, p < 0.0001) (Figure 5.2). Additionally, there was a significant main effect of GDNF treatment ($F_{1,79}$ = 71.096, p < 0.0001) with a general increase in locomotor activity in GDNF-treated groups of both genotypes (weeks 1-4 in *Gdnf*$^{+/-}$ mice and weeks 1-3 in WT mice). There was also a significant main effect of time ($F_{3,79}$ = 32.315, p < 0.0001) with both genotypes (GDNF-treated) demonstrating an initial increase in locomotor activity that declined over the 4-week testing period.

Mice treated with GDNF (n = WT: 5 *Gdnf*$^{+/-}$*:* 7) or vehicle (n = WT: 4 *Gdnf*$^{+/-}$*:* 7) were tested for stimulated motor activity following nomifensine (4-weeks after GDNF treatment). Data were analyzed using a genotype x treatment 1 (GDNF/vehicle) x treatment 2 (nomifensine/saline) three-way ANOVA. As we have previously shown (Littrell*, et al.*, 2010), the administration of nomifensine increased locomotor activity in both genotypes treated with GDNF or vehicle compared to saline administration *(*$F_{1,38}$= 158.057, p < 0.0001). There was a significant interaction between treatment 1 (GDNF/vehicle) and treatment 2 (nomifensine/saline) ($F_{1,38}$ = 4.263, p = 0.0458) with GDNF-treated groups showing a greater response to nomifensine in both genotypes (Figure 5.2). These data suggest a synergistic effect of combining GDNF treatment with nomifensine to increase locomotor activity.

Tyrosine Hydroxylase Immunohistochemistry

The number of DA neurons (TH-positive neurons) was determined in *Gdnf*$^{+/-}$ (n = 6) and WT (n = 4*)* mice after GDNF treatment (Figure 5.3 A). Four weeks after striatal GDNF administration there was a significant effect of treatment on the number of DA neurons in the SN [$F_{3,16}$ = 31.70 p < 0.0001, one-way ANOVA (Figure 5.3 B)]. Bonferroni *post hoc* analysis indicates *Gdnf*$^{+/-}$ mice had fewer TH-positive neurons compared to WT mice (both vehicle-treated) [mean ± SEM, number of neurons; WT: 4209 ± 150; *Gdnf*$^{+/-}$: 2841 ± 104; ***p < 0.001] as determined previously (Boger*, et al.*, 2006). There was also a significant increase in the number of TH-positive neurons after GDNF treatment in *Gdnf*$^{+/-}$ mice [mean ± SEM, number of neurons; Vehicle: 2841 ± 104, GDNF: 3852 ± 132; ***p < 0.001] but no significant difference in TH-positive neurons between treatments in WT mice [p > 0.05]. There was a significant difference between genotypes treated with GDNF [mean ± SEM, number of neurons; WT: 4600 ± 171; *Gdnf*$^{+/-}$: 3852 ± 132; **p <

0.001] and a significant difference between WT mice treated with GDNF and *Gdnf*$^{+/-}$ mice treated with vehicle [***$p < 0.001$].

Dopamine Release – Electrochemical Studies

Evoked DA release by local application of high-potassium solution was determined for WT and *Gdnf*$^{+/-}$ mice after striatal treatment with GDNF (Figure 5.4). Data were analyzed using two-tailed unpaired *t* tests to compare treatment effects within genotypes. A significant decrease (*$p = 0.0196$, $t_{11} = 2.73$) in release amplitude after GDNF treatment was apparent in the dorsal striatum of the rostral-medial recording site in WT mice [Figure 5.4A, mean ± SEM (μM); Vehicle: 0.78 ± 0.28 ($n = 3$), GDNF: 0.26 ± 0.071 ($n = 10$), *n* represents the number of peaks].

Tissue Neurochemical Content

Neurochemical content was investigated to determine if there were changes in tissue levels of DA or DA-metabolites: DOPAC and HVA. Neurochemical levels were analyzed using a two-tailed unpaired *t* test comparing treatment groups within genotypes for rostral (AP+1) and caudal (AP+0.1) brain sections (Table 5.1). Two samples were obtained from each brain section in each animal. In rostral sections, there was as a significant increase in DA content in non-littermate/non-transgenic C57/BL6 mice treated with GDNF [*$p = 0.0183$]. In caudal sections, there was a significant increase in DOPAC in WT mice treated with GDNF [*$p = 0.016$] and C57/BL6 mice treated with GDNF [*$p = 0.0386$].

Discussion

GDNF Protein Levels are Increased by Striatal GDNF Treatment

Delivery of GDNF to the striatum of *Gdnf*$^{+/-}$ mice led to an increase (40%) in striatal GDNF protein levels compared to vehicle-treated *Gdnf*$^{+/-}$ mice (4-weeks after treatment). A comparable difference in GDNF protein was not seen in treated WT mice, although there was an increase (statistically insignificant) in GDNF protein. It is unknown how processing or clearance of exogenous GDNF may be altered in *Gdnf*$^{+/-}$ mice. It is possible that a higher dose of GDNF is necessary to result in detectable changes in WT mice, which do not demonstrate protein reductions (Boger*, et al.*, 2006). There was also a significant difference in GDNF-treated WT and *Gdnf*$^{+/-}$ mice with more GDNF protein in

WT mice. Thus, treatment with GDNF led to an increase in striatal protein only in *Gdnf*$^{+/-}$ mice. The effective striatal protein levels were comparable to WT (vehicle) animals but did not reach levels equivalent to that seen in GDNF-treated WT mice. Thus, protein levels in GDNF-treated *Gdnf*$^{+/-}$ mice were restored to levels comparable to WT counterparts ($p > 0.05$). A significant reduction (36%) in GDNF protein in the vehicle-treated *Gdnf*$^{+/-}$ mice compared to vehicle-treated WT mice corroborates previous reports of protein reductions in naïve *Gdnf*$^{+/-}$ mice (Boger*, et al.*, 2006). Qualitative TH-ir histological studies suggest that striatal GDNF treatment increased staining intensity of GDNF in the SN of both WT and *Gdnf*$^{+/-}$ mice. Expression of GDNF is highest in the developing striatum (Stromberg*, et al.*, 1993), however, retrograde transport to the SN (Barroso-Chinea*, et al.*, 2005) is suggested to play a critical role in activation of the nigral localized GDNF receptor - GFRα1 (Quartu*, et al.*, 2007). Indeed, retrograde transport of GDNF by striatal DA terminals has been demonstrated previously in animal models – occurring within 24 hours after striatal delivery (Ai*, et al.*, 2003; Lapchak*, et al.*, 1997b). In these studies retrograde transport processes are suggested by the increase in GDNF-staining intensity in the SN. Although increased receptor activation in the SN after GDNF treatment is likely, this possibility requires further investigation.

Locomotor Behavior Enhanced by GDNF Treatment

As shown previously *Gdnf*$^{+/-}$ mice demonstrated reduced basal locomotor activity and enhanced sensitivity to nomifensine-stimulated locomotor activity compared to WT counterparts (Boger*, et al.*, 2006; Littrell*, et al.*, 2010) (see Nomifensine-Induced Locomotor Activity). Treatment with GDNF increased spontaneous locomotor activity in both genotypes, an effect that appears to diminish over time. *Gdnf*$^{+/-}$ mice displayed a more sustained increase in activity with greater activity than WT mice (also treated with GDNF) at 3- and 4-weeks. Importantly, these studies show that GDNF treatment can restore behavioral deficits in middle-aged *Gdnf*$^{+/-}$ mice to comparable activity levels as WT counterparts. It is hypothesized that *Gdnf*$^{+/-}$ mice demonstrate increased sensitivity to GDNF treatment due to chronic GDNF depletion and compensatory increases in GFRα1-receptor expression – a finding demonstrated in aged rats (Pruett and Salvatore, 2010). Because changes in GFRα1-receptor expression have been correlated with TH-ir (Pruett and Salvatore, 2010), GFRα1-receptor expression has been suggested to regulate nigral DA tissue content. Thus, changes in DA availability may also contribute to the enhancing effects of GDNF, which are sustained in *Gdnf*$^{+/-}$ mice.

The nomifensine-induced increase in locomotor activity was seen in all genotypes and treatment groups and is consistent with the known pharmacological and behavioral effects of the DAT inhibitor (Altar and Marshall, 1988). Sensitivity to nomifensine was increased in GDNF-treated groups of both genotypes compared to vehicle-treated groups of the same genotype. This enhanced effect may be attributed to increased DA-neuron function in GDNF-treated groups as stated above. Indeed both genotypes showed increased TH-ir (See Tyrosine Hydroxylase Immunohistochemistry) that may result in increased: DA content, DA-receptor activation and motor activity (Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996). The enhanced activity in GDNF-treated *Gdnf*$^{+/-}$ mice *versus* GDNF-treated WT mice may be due to increased DA receptors (Kelly*, et al.*, 1998) (see Striatal Dopamine D2-receptor mRNA and Protein Expression) or compensatory increases in sensitivity to GDNF through upregulation of GFRα1 receptors as discussed above.

We expected these behavioral findings to be accompanied with increases in DA and DA-metabolite neurochemical levels as shown in previous studies using GDNF in rats (Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996). However, the neurochemical studies are not in agreement with behavioral measures. We believe the disagreement with neurochemical studies is due to factors described below (see GDNF Treatment Increases Neurochemical Levels in C57/Bl6 Mice and Non-Transgenic WT Mice). It is possible that the increased TH-ir in the SN does not cause neurochemical changes at striatal terminals. Alternatively, DA and DA-metabolite neurochemistry may be enhanced at the cell bodies in the SN only. Nigral neurochemical effects and somatodendritic receptor modulation have also been correlated with improved behavioral measures (Bergquist*, et al.*, 2003) and GDNF treatment (Hebert and Gerhardt, 1997; Hebert*, et al.*, 1996; Hoffer*, et al.*, 1994). Although neurochemical levels were not directly determined in the SN, the increase in TH-ir staining in the SN supports this hypothesis.

GDNF Treatment Increased Tyrosine Hydroxylase Staining

Consistent with prior studies establishing histological differences between genotypes (Boger*, et al.*, 2006), there was a decrease (48%) in number of TH-positive neurons in vehicle-treated *Gdnf*$^{+/-}$ mice *versus* vehicle-treated WT mice. Treatment with GDNF increased (36%) the number of TH-positive neurons in the SN of *Gdnf*$^{+/-}$ mice compared to vehicle treated *Gdnf*$^{+/-}$ mice. While the number of TH-positive neurons is substantially lower than GDNF-treated WT mice, GDNF-treated *Gdnf*$^{+/-}$ mice appear to

demonstrate equivalent values compared with WT (vehicle) mice. Thus, GDNF treatment in *Gdnf*$^{+/-}$ mice restored the number of TH-positive neurons to comparable levels as vehicle-treated WT counterparts ($p > 0.05$). The increase in TH-positive neurons supports increased nigrostriatal DA-neuron function, which we would expect to be accompanied with enhanced neurochemical levels and accompanying behavioral improvements. Although an increase in staining of TH – the rate-limiting enzyme in DA synthesis – is encouraging, investigation of other aspects of TH activity and DA-neuron function are necessary to more clearly determine the effect(s) of GDNF treatment. Of note, GDNF treatment has been shown to increase TH levels without concomitant increases in DA neurochemical levels (Yang*, et al.*, 2009). The phosphorylation state of TH may also determine effective changes in neurochemical levels and has been shown to be modulated by GDNF (Salvatore*, et al.*, 2004; Yang*, et al.*, 2009). It is currently unclear if the TH-ir increase is a result of increased numbers of TH-positive neurons or upregulation of TH expression, both of which may be modulated by GDNF and GDNF-receptor presence/activation (Cao*, et al.*, 2010; Pruett and Salvatore, 2010).

Interestingly, GDNF-treated WT mice did not demonstrate increased TH-positive neurons *versus* vehicle-treated WT counterparts ($p > 0.05$). Although enhancement of some DA neurochemical levels has been reported in the intact nigrostriatal pathway of young (Hebert*, et al.*, 1996) and aged rodents (Hebert and Gerhardt, 1997), prior animal studies investigating TH-ir after GDNF treatment have yielded inconsistent results. A decrease in TH-ir after GDNF delivery to the intact nigrostriatal pathway of the rat (Rosenblad*, et al.*, 2003) has been reported, but another study in the aged primate reported an increase in number of TH-positive neurons after GDNF delivery (Palfi*, et al.*, 2002). It has been proposed that GDNF treatment causes a transient phenotypic shift – evident by an initial downregulation of TH. In parkinsonian rats (6-OHDA striatal lesioned) treated with GDNF, an initial decline in TH-ir has been demonstrated with a delayed increase in TH-positive neurons (8 weeks after GDNF treatment (Cohen*, et al.*, 2011)). Although the current studies were modeled after *in vivo* studies that addressed similar neurochemical and behavioral parameters in the aged rat (Hebert and Gerhardt, 1997), it is possible that the effect of GDNF on TH may require a longer time course than examined here. The time course may affect the results seen in both genotypes. The effect on TH may develop later for WT mice and be more pronounced in *Gdnf*$^{+/-}$ mice at later time points. Comparison of parkinsonian rats and the current mice studies presents with confounding factors i.e. ongoing neurodegeneration in striatal lesion models, which

implicates both neuro-restorative and -protective mechanisms (Sauer and Oertel, 1994). We postulate that *Gdnf*$^{+/-}$ mice, demonstrating a chronic GDNF depletion, may be more sensitive to GDNF supplementation due to a compensatory increase in surface expression of GDNF receptors (Kozlowski*, et al.*, 2004; Pruett and Salvatore, 2010) or other mechanisms, which require further investigation.

Generally*,* the TH studies mirror results from GDNF protein quantification (see GDNF Protein Levels). Specifically, no significant differences in protein levels were seen between GDNF-treated WT and vehicle-treated WT mice. Again, it may be necessary to increase the treatment dose to detect significant changes in protein levels and TH-ir in WT mice because WT mice do not demonstrate deficits in GDNF protein or TH-ir.

GDNF Treatment Decreased Dopamine Release

The validity of DA signals obtained from electrochemical studies has been questioned due to the dramatic difference in release amplitude values from previous studies in naïve animals of the same age and genotypes (see Studies of Dopamine Release). Due to these initial observations, non-littermate C57/BL6 mice (see Delivery of GDNF) were also used in electrochemical studies, which failed to produce appropriate signals for analysis (data not shown). Because observations in naïve mice show greater striatal DA release than in the current studies, it is suspected that injection of GDNF into the striatum may interfere with this recording technique. Thus, discussion of the findings of DA release should be considered with these important caveats.

Interestingly, GDNF treatment significantly (67%) decreased evoked DA release in the dorsal striatum of WT mice. Dopamine-receptor agonists have been shown to positively influence GDNF expression (Ohta*, et al.*, 2003). It is possible that GDNF also modulates DA release – effectively modulating DA-receptor activation. Thus, GDNF supplementation in WT mice may cause a downregulation of DA release. The decreased release does not appear to be accompanied with changes in TH because WT mice did not demonstrate any detectable differences in TH-ir in the SN, although there may be changes in TH activity (Salvatore*, et al.*, 2004) that are not apparent in these histochemical analyses. Analysis of whole tissue neurochemical levels is discussed below (see GDNF Treatment Increases Neurochemical Levels in C57/Bl6 mice and Non-transgenic WT Mice). This finding demonstrates a differential response to GDNF in WT *versus Gdnf*$^{+/-}$ counterparts with the latter lacking detectable differences in DA release after GDNF treatment. Compensatory changes in GDNF-receptor expression in *Gdnf*$^{+/-}$

mice are expected and may contribute to the differential release findings – causing a dose-response shift between *Gdnf*$^{+/-}$ and WT mice.

GDNF Treatment Increases Neurochemical Levels in C57/Bl6 Mice and Non-Transgenic WT Mice

Based on prior studies showing heterogeneous function of DA terminals in the dorsal and ventral striatum of *Gdnf*$^{+/-}$ mice, striatal sections were subdivided into dorsal and ventral samples for HPLC-EC analysis of neurochemical content. The resulting samples were below the ideal size (weight) to determine neurochemical levels (personal communication, Wayne Cass, Ph.D.) The following neurochemical findings should be considered with this caveat.

Dopamine neurochemical content in the anterior striatum was significantly increased (63%) after GDNF treatment in non-littermate C57/BL6 mice. In the posterior striatum, DOPAC levels were increased in GDNF-treated C57/BL6 mice (41%) as well as GDNF-treated WT mice (56%). A lack of effect on DA neurochemistry in either striatal section in *Gdnf*$^{+/-}$ mice is surprising because of the increase in TH-ir in the SN. We would expect changes in TH to cause increases in DA or DA-metabolite levels. Additionally, WT mice, which did not demonstrate increased TH staining intensity, did demonstrate increased striatal DA and DOPAC levels. The relationships among GDNF, TH expression and neurochemical levels have not been clearly determined. Some studies have shown decreased TH expression without parallel changes in neurochemical content with GDNF treatment (Rosenblad*, et al.*, 2003).

Consideration of incongruent histological and neurochemical findings (GDNF Treatment Increased Tyrosine Hydroxylase Staining) leads us to believe that the small sample size (weight) was a major interfering factor in accurately determining tissue weight and neurochemical content. Therefore, the calculation of neurochemical levels (ng/g gram wet weight of tissue) is likely inaccurate.

Conclusions

Long-term changes in nigrostriatal DA-neuron function related to GDNF depletion are suspected due to the critical role of GDNF in developmental processes (Granholm*, et al.*, 2000; Stromberg*, et al.*, 1993). These studies further investigated the functional abnormalities of DA neurons and behavioral deficits, which appear to be responsive to trophic factor supplementation. Furthermore, sensitivity to trophic factor treatment

appears to be influenced by a chronic and partial GDNF depletion with WT and *Gdnf*$^{+/-}$ mice demonstrating altered responses to equivalent doses of GDNF. Altogether, these studies suggest that regulation of endogenous trophic factors contributes to age-associated dysfunction of the nigrostriatal pathway and behavioral impairments and these deficits are potential targets for trophic factor treatment.

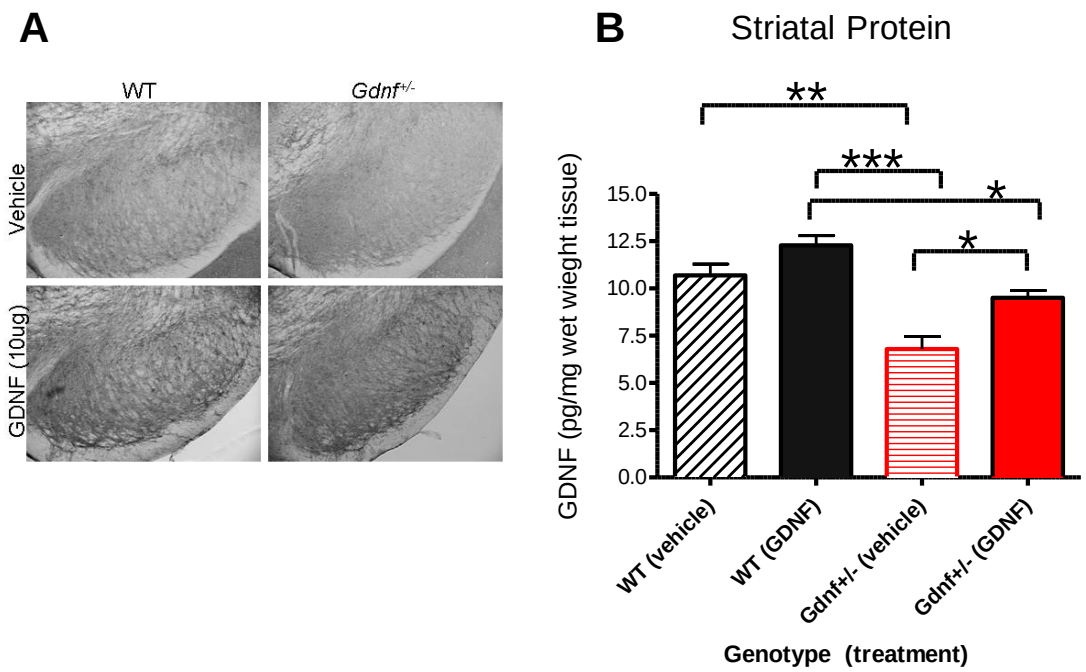

Figure 5.1 GDNF Protein in the Substantia Nigra and Striatum
Tissue sections through the SN were stained for GDNF-ir (4 weeks after striatal treatment of GDNF or vehicle). Representative sections show increased staining intensity for GDNF in both genotypes treated with GDNF *versus* vehicle treatment (A). GDNF protein levels were quantified by ELISA in striatal tissues (B). GDNF protein levels in the striatum were averaged within genotypes of each treatment group. Vehicle-treated *Gdnf*$^{+/-}$ mice had decreased GDNF protein levels *versus* vehicle-treated WT (**p < 0.01) and GDNF-treated WT (***p < 0.001) mice. There was significant increase in GDNF protein in *Gdnf*$^{+/-}$ mice treated with GDNF *versus* vehicle (*p < 0.05). In addition, GDNF-treated *Gdnf*$^{+/-}$ mice demonstrated decreased GDNF protein levels compared to GDNF-treated WT mice (*p < 0.05). Data are shown as mean ± SEM. Data were analyzed by a one-way ANOVA with Bonferroni's *post hoc* tests.

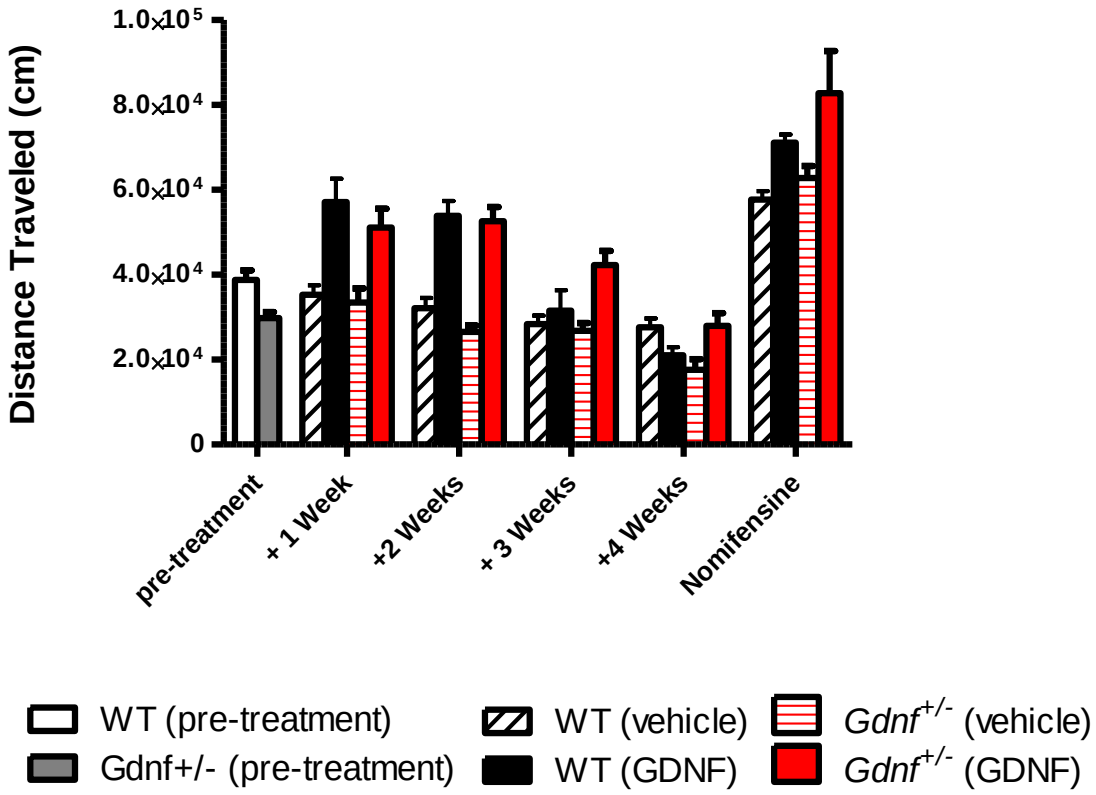

Figure 5.2 Spontaneous and Nomifensine-induced Locomotor Activity in GDNF-treated *Gdnf*$^{+/-}$ and WT Mice

Total distance traveled was measured prior to treatment and weekly after striatal treatment with GDNF or vehicle. Spontaneous motor activity was measured at all time points. Stimulated motor activity following nomifensine (7.5 mg/kg i.p.) was measured 4-weeks after treatment. Pre-treatment locomotor activity was analyzed using a two-tailed unpaired *t* test. *Gdnf*$^{+/-}$ mice displayed a significant decrease (***p* = 0.0022; t_{20} = 3.515) in locomotor activity *versus* WT mice. Spontaneous locomotor activity following treatment was analyzed using a 2 (genotype) x 2 (treatment) x 4 (intervals) mixed-factor ANOVA with repeated measures. There was a significant effect of GDNF treatment ($F_{1,79}$ = 71.096, $p < 0.0001$) with greater locomotor activity in GDNF-treated groups. There was also a significant effect of time ($F_{3,79}$ = 32.315, $p < 0.0001$) with both GDNF-treated genotypes exhibiting an increase in locomotor activity after treatment, which diminished over the 4-week testing period. There were also significant interactions: genotype x treatment ($F_{1,79}$= 5.114, p = 0.0265) and treatment x time ($F_{3, 79}$ = 9.255, $p < 0.0001$). Nomifensine-stimulated locomotor activity data was analyzed using a genotype x

treatment 1 (GDNF/vehicle) x treatment 2 (nomifensine/saline) three-way ANOVA. There was a significant effect of GDNF treatment ($F_{1,38}$ = 6.764, p = 0.0132) with greater stimulated activity in GDNF-treated groups than vehicle-treated groups of both genotypes. There was a significant effect of nomifensine ($F_{1,38}$= 158.057, p < 0.0001) with greater activity following nomifensine compared to saline (spontaneous locomotor activity at 4 weeks) in all genotypes and treatment groups. Additionally, there was a significant interaction between treatment 1 (GDNF/vehicle) and treatment 2 (nomifensine/saline) ($F_{1,38}$ = 4.263, p = 0.0458) with GDNF-treated groups showing a greater response to nomifensine (both genotypes). All data are shown as mean ± SEM.

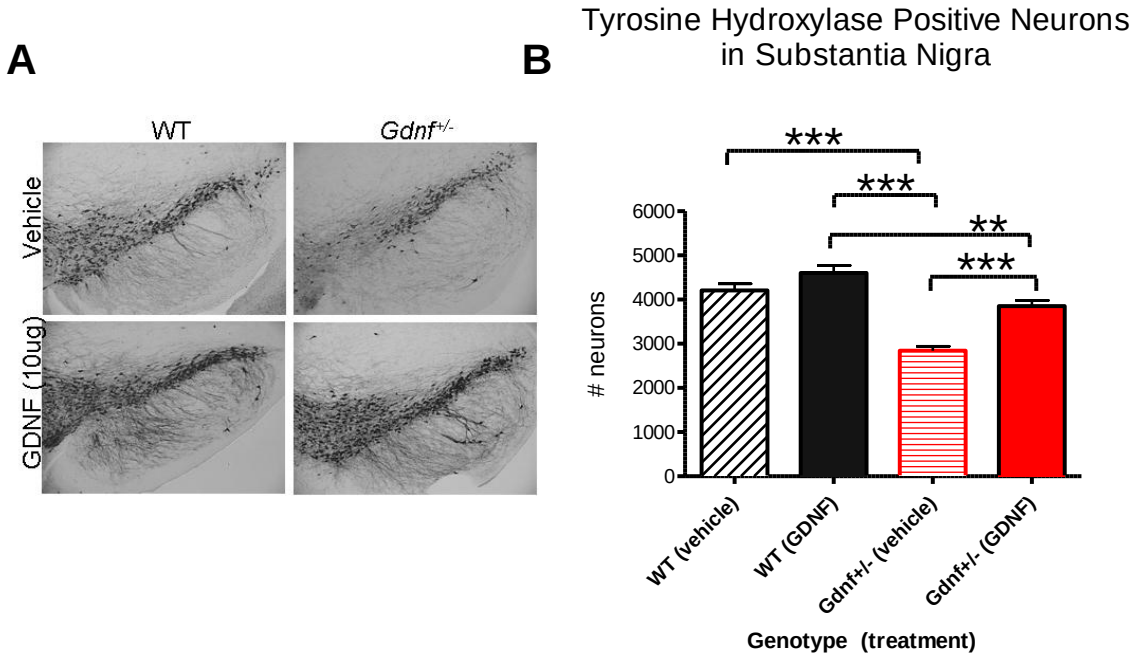

Figure 5.3 Tyrosine Hydroxylase Immunoreactivity (TH-ir) in the Substantia Nigra
Tissue sections through the SN were stained for TH-ir (4 weeks after striatal treatment with GDNF or vehicle). Representative sections (A) show increased numbers of TH-positive neurons and increased fiber density in both genotypes treated with GDNF *versus* vehicle treatment. The number of TH-positive neurons in the SN was averaged for treatment groups of each genotype (B). *Gdnf*$^{+/-}$ (vehicle) mice had fewer TH-positive neurons *versus* WT (vehicle) (***$p < 0.001$) and WT (GDNF treated) (***$p < 0.001$) mice. There was a significant increase in number of neurons in *Gdnf*$^{+/-}$ mice treated with GDNF (***$p < 0.001$, *versus* vehicle). Additionally, GDNF-treated *Gdnf*$^{+/-}$ mice had fewer TH-positive neurons compared to GDNF-treated WT mice (**$p < 0.01$). Data are shown as mean ± SEM. Data were analyzed by a one-way ANOVA with Bonferroni's *post hoc* tests.

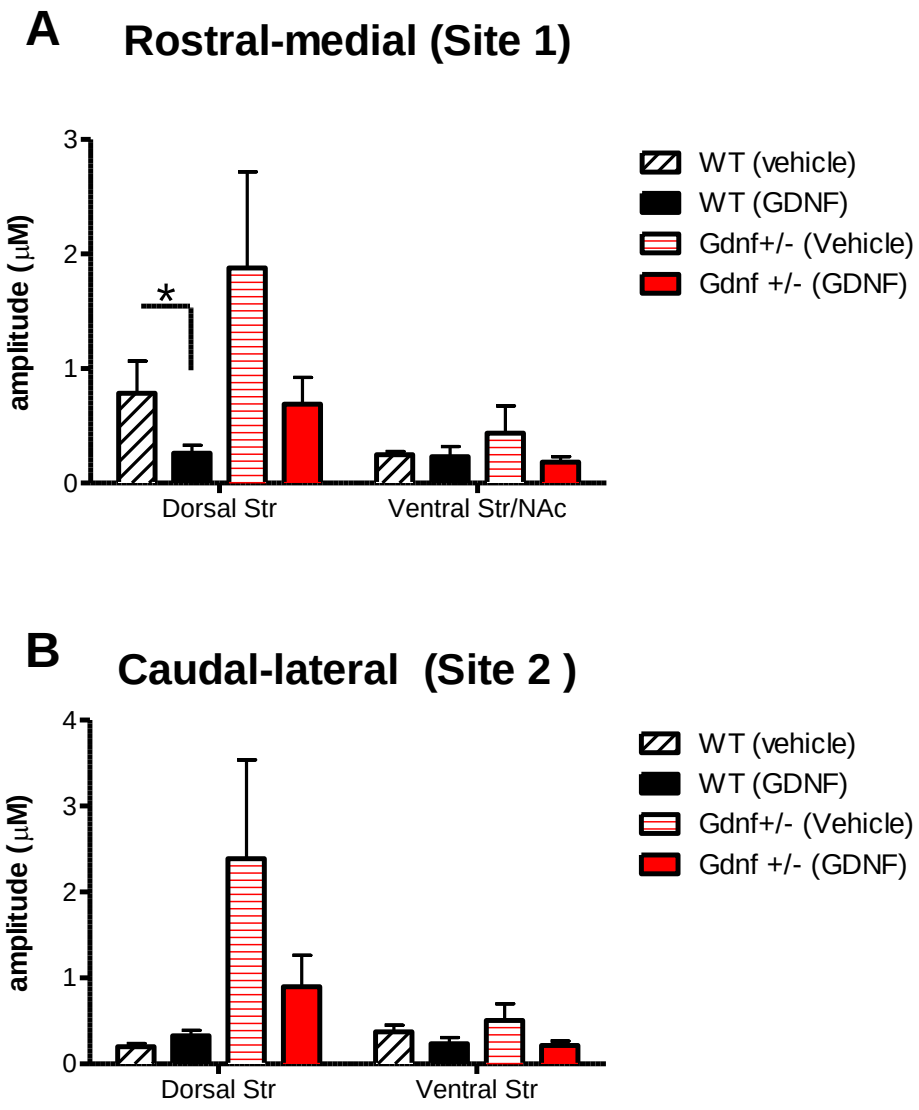

Figure 5.4 Amplitude of Evoked-Dopamine Signals in GDNF-treated *Gdnf*$^{+/-}$ Mice
A potassium chloride solution (120 mM) was applied locally (75-150 nL) in order to measure DA release in the striatum (4-weeks after striatal treatment with GDNF or vehicle). The resulting amplitude from striatal sub-regions was averaged and analyzed to compare treatment groups. In the rostral-medial recording site (A) there was a significant (*p = 0.0196) decrease in release amplitude in the dorsal striatum of WT mice. In the caudal-lateral recording site (B) there were no significant changes (p > 0.05) in DA release after GDNF treatment for either *Gdnf*$^{+/-}$ or WT mice. Data are shown as mean ± SEM and were analyzed by a two-tailed unpaired t test comparing treatment of like genotypes within striatal sub-regions.

Table 5.1 Tissue Neurochemical Content after Treatment with GDNF

Genotype	Treatment	Striatal Region	DA	DOPAC	HVA	DOPAC:DA x10^3	HVA:DA x10^3	(DOPAC+HVA):DA x10^3
WT	Vehicle	AP: +1.0	14115 ± 1901	3403 ± 416	2925 ± 389	271.5 ± 43.3	222.0 ± 33.8	493.6 ± 65.4
WT	GDNF	AP: +1.0	11113 ± 998	4228 ± 473	2026 ± 128	347.4 ± 47.6	180.7 ± 14.6	552.8 ± 48.3
Gdnf+/-	Vehicle	AP: +1.0	13483 ± 706	3705 ± 499	2550 ± 206	298.6 ± 30.1	199.5 ± 13.9	468.0 ± 36.2
Gdnf+/-	GDNF	AP: +1.0	15583 ± 1575	4307 ± 504	2824 ± 305	293.4 ± 35.5	195.8 ± 17.0	476.8 ± 43.4
C57/Bl6	Vehicle	AP: +1.0	7820 ± 1547, *n* = 6	1583 ± 349	1983 ± 381	209.7 ± 23.2	294.7 ± 65.1	504.7 ± 86.5
C57/Bl6	GDNF	AP: +1.0	21061 ± 5112*, *n* = 4 *t*(8) = 2.995 *p* = 0.0183	2504 ± 134	2136 ± 456	142.5 ± 32.6	117.5 ± 32.9	260.0 ± 60.9
WT	Vehicle	AP: +0.1	5933 ± 1280	2422 ± 415, *n* = 8	2327 ± 156	367.0 ± 45.1	311.8 ± 45.8	700.7 ± 71.4
WT	GDNF	AP: +0.1	9186 ± 1077	3786 ± 275*, *n* = 8 *t*(14) = 2.740; *p* = 0.016	2526 ± 219	430.5 ± 38.3	270.7 ± 21.3	660.2 ± 63.8
Gdnf+/-	Vehicle	AP: +0.1	9074 ± 1655	3236 ± 453	2162 ± 237	390.7 ± 43.7	262.5 ± 57.8	520.9 ± 24.4
Gdnf+/-	GDNF	AP: +0.1	9538 ± 1114	3043 ± 398	2251 ± 164	362.9 ± 36.5	253.4 ± 41.3	595.4 ± 49.1
C57/Bl6	Vehicle	AP: +0.1	5243 ± 1345	1347 ± 275, *n* = 6	1613 ± 215	292.2 ± 29.4	388.8 ± 105	562.8 ± 71.3
C57/Bl6	GDNF	AP: +0.1	8766 ± 550	2281 ± 198*, *n* = 4 *t*(8) = 2.472 *p* = 0.0386	2475 ± 322	260.0 ± 15.3	280.3 ± 25.9	540.3 ± 21.2

Data shown as mean ± SEM (ng/g wet weight of tissue). Analyzed by a two-tailed unpaired *t* test comparing treatment groups within genotypes and striatal regions.

Chapter Six: Final Conclusions

These studies highlight the potential of GDNF and similar molecules to modulate DA-neuron function (Cowen and Gavazzi, 1998). The current findings are relevant to the neurodegenerative processes involved in PD as well as brain aging (McGeer*, et al.*, 1988; Volkow*, et al.*, 1998b). The ability of the nigrostriatal pathway to combat neurodegenerative processes is highlighted by the requirement of a severe loss of DA innervation to manifest motor impairment in Parkinson's disease (Bernheimer*, et al.*, 1973). Thus, the nigrostriatal pathway exhibits a high degree of plasticity and compensatory capacity that can be exploited by trophic factor supplementation (Peterson and Nutt, 2008). Although there is conflicting evidence regarding the definitive role of GDNF in aging, Parkinson's disease and experimental models of DA-neuron injury (Backman*, et al.*, 2006; Jenner and Olanow, 1998; Nakajima*, et al.*, 2001; Smith*, et al.*, 2003), these studies provide support for the importance of endogenous GDNF in brain function. Finally, supplementation with trophic factors and/or phenotypic-mimetic peptides (e.g. DNSPs) is a favorable approach for the treatment of nigrostriatal dysfunction.

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Scholastic and Professional Honors

2002-2006	Annual merit based scholarship: Brown Colonel Scholar. Centre College, Danville, Kentucky.
2006-2007	Academic Fellowship: Graduate School Academic Year Fellowship. University of Kentucky, Lexington, Kentucky.
2009-2010	Predoctoral Fellowship: NIH Training Grant IT32 DA022738, "Therapeutic Strategies for Neurodegeneration". University of Kentucky, Lexington Kentucky.
2010	Invited Speaker. Dynamic Changes in Dopamine Uptake in *Gdnf*$^{+/-}$ Mice. Biochemistry and Molecular Biology. Seminar Series. Centre College, Danville, KY
2010-	Predoctoral Fellowship: NIH Training Grant 5T32AG000242-14, "Cellular and Molecular Basis of Brain Aging". University of Kentucky, Lexington Kentucky.

Publications

Manuscripts

2010 Bradley, L.H., J. Fuqua, A. Richardson, J. Turchan-Cholewo, Y. Ai, K.A. Kelps, J.D. Glass, X. He, Z. Zhang, R. Grondin, **O.M. Littrell**, P. Huettl, F. Pomerleau, D.M. Gash, and G.A. Gerhardt. Dopamine neuron stimulating actions of a GDNF propeptide. *PLoS One*. 5:e9752.

2010 **Littrell, O.M**., F. Pomerleau, P. Huettl, S. Surgener, J.F. McGinty, L.D. Middaugh, A.C. Granholm, G.A. Gerhardt, and H.A. Boger. Enhanced dopamine transporter activity in middle-aged Gdnf heterozygous mice. *Neurobiol Aging*.

2010 Richardson, A., **O.M. Littrell**, S. Challman, and P. Stein. Using Text Messaging in an Undergraduate Nursing Course. J Nurs Educ:1-6

Abstracts

2007 J.M. Hinzman, **O.M. Littrell**, P. Huettl, F. Pomerleau, C.G. Yu, J.W. Geddes, G.A. Gerhardt. Second-by-Second Recordings of l-glutamate in a Rat Spinal Cord and Injury Model Using Enzyme-Based Microelectrode Arrays. Neuroscience Day **-** Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2008 **O.M. Littrell**, J.L. Fuqua, A. Richardson, E.R. Hascup, F. Pomerleau, P. Huettl, D.M. Gash, and G.A. Gerhardt. Characterization of the effects of Dopamine Neuron Stimulating Peptide (DNSP-11) on Dopamine Neuron Function. Monitoring Molecules in Neuroscience. 12th International Conference on *In Vivo* Methods. Vancouver, British Columbia, Canada.

2008 **O.M. Littrell**, J.L. Fuqua, E.R. Hascup, P. Huettl, F. Pomerleau, G.A. Gerhardt*. In vivo* Effects of a Novel Dopamine Neuron Stimulating peptide (DNSP-11). Neuroscience Day - Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2009 **O.M. Littrell**, H. Boger, P. Huettl, F. Pomerleau, J.E. Quintero, A.-Ch. Granholm, and G.A. Gerhardt. Twelve month old glial cell derived neurotrophic factor heterozygous mice show increased dopamine release and dopamine uptake in the striatum. 39th annual meeting - Society for Neuroscience. Chicago, IL.

2009 **O.M. Littrell**, H.A. Boger, P. Huettl, F. Pomerleau, A.E. Granholm, G.A. Gerhardt*. In vivo* changes in dopamine release and uptake in mice with a

partial GDNF deletion. Center for Clinical and Translational Science Conference. Lexington, KY.

2009 **O.M. Littrell**, H.A. Boger, P. Huettl, F. Pomerleau, A.E. Granholm, G.A. Gerhardt. *In vivo* changes in dopamine release and uptake in mice with a partial GDNF deletion. Neuroscience Day – Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2009 J.S. Mason, **O.M. Littrell**, P. Huettl, F. Pomerleau, G.A. Gerhardt. *In vivo* effects of effects of dopamine neuron stimulating peptide 11 in the rat striatum using chronoamperometry. Neuroscience Day – Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2010 **O.M. Littrell**, R.J. Chang, P. Huettl, F. Pomerleau, J.E. Quintero, G.A. Gerhardt. Intranigral Dopamine Neuron Stimulating Peptide-11 Treatment Enhances Dopamine Neuron Function. Center for Clinical and Translational Science Conference. Lexington, KY.

2010 **O.M. Littrell**, R.J. Chang, P. Huettl, F. Pomerleau, J.E. Quintero, G.A. Gerhardt. Intranigral Dopamine Neuron Stimulating Peptide-11 Treatment Enhances Dopamine Neuron Function. Neuroscience Day – Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2010 **O.M. Littrell**, J.L. Fuqua, A.D. Richardson P. Huettl, F. Pomerleau, J.E. Quintero, L.H. Bradley, D.M. Gash, G.A. Gerhardt. A new proGDNF peptide – dopamine neuron stimulating peptide-5 – enhances dopamine neuron function in vitro and in vivo. 40th annual meeting - Society for Neuroscience. San Diego, CA.

2010 L.H. Bradley, J. Fuqua, A. Richardson, J. Turchan-cholewo, Y. Ai, KA Kelps, J.D. Glass, X. HE, Z. Zhang, R. Grondin, **O.M. Littrell**, P. Huettl, F. Pomerleau, D.M. Gash, G.A. Gerhardt. Antiparkinsonism actions of dopamine neuron stimulating peptide-11. 40th annual meeting - Society for Neuroscience. San Diego, CA.

2011 **O.M. Littrell**, J.L. Fuqua, A.D. Richardson P. Huettl, F. Pomerleau, J.E. Quintero, L.H. Bradley, D.M. Gash, G.A. Gerhardt. A GDNF related peptide – dopamine neuron stimulating peptide-5 – enhances dopamine neuron function. Neuroscience Day – Bluegrass Chapter for the Society of Neuroscience. Lexington, KY.

2011 **O.M. Littrell**, J.L. Fuqua, A.D. Richardson P. Huettl, F. Pomerleau, J.E. Quintero, L.H. Bradley, D.M. Gash, G.A. Gerhardt. A GDNF related peptide – dopamine neuron stimulating peptide-5 – enhances dopamine neuron function. Center for Clinical and Translational Science Conference. Lexington, KY.